Controlling Foodborne Microorganisms in Alfalfa Sprouts

by

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Abstract

Raw sprouts are consistently found as the source of produce related foodborne illness outbreaks. Research and past experiences illustrate that intervention methods currently used in the sprout industry are occasionally ineffective. In addition, possible alternatives proven to reduce microbial loads, oftentimes negatively affect the physical or economical attributes of the product. The use of MC (1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone), an extremely stable monochlorinated *N* – halamine that possesses high antimicrobial activity, may serve as a plausible alternative without the impractical drawbacks. The purpose of this study is to evaluate the antimicrobial activity of MC, to investigate its effectiveness at reducing foodborne pathogens on alfalfa sprouts, and to determine if MC is a commercially applicable intervention strategy for the control of pathogens in raw sprouts. The antimicrobial test of MC illustrated that 0.05% MC solution was able to kill *Salmonella, Escherichia coli* O157:H7, and *Listeria monocytogenes* completely at 10^6 CFU/mL. Based on this finding, we investigated the antimicrobial activity of MC on inoculated sprouts. Results showed that MC was able to reduce bacterial populations, however, the reductions were minimal due to the physical structure of alfalfa sprouts sheltering pathogens from the biocidal effects of the compound. This application of MC was not found to compromise the economical integrity of mature alfalfa sprouts, however, further research is warranted to discover how to effectively inactivate sheltered pathogens in sprouts to enhance their microbial safety.

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List of Abbreviations

BP	Baird-Parker Agar
BPB	Butterfield's Phosphate Buffer
Br	Bromine
BSA	Bismuth Sulfite Agar
CDC	US Center for Disease Control and Prevention
CFU	Colony Forming Unit
Cl	Chlorine
ClO_2	Chlorine Dioxide
^{60}Co	Cobalt-60
^{137}Cs	Cesium-137
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EHEC	Enterohemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Entertoxigenic *Escherichia coli*
FDA	US Food and Drug Administration
FSIS	Food Safety and Inspection Service
GAP	Good Agricultural Practices
GMP	Good Manufacturing Practices

GRAS	Generally Recognized As Safe
HACCP	Hazard Analysis and Critical Control Points
HE	Hektoen Enteric Agar
kGy	kilograys
LD_{50}	Value of the oral dose that produces lethality in 50% of test animals
L. monocytogenes	*Listeria monocytogenes*
MAC	MacConkey Agar
MC	1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone
MOX	Modified Oxford Medium
N	Normal
NA	Nalidixic Acid
NACMCF	National Advisory Committee on Microbiological Criteria for Foods
NOEL	No Observed Effect Level
OD	Optical Density
ppm	Parts Per Million
RTE	Ready-to-Eat
SAS	Statistical Analysis Software
S. aureus	*Staphylococcus aureus*
S. Enteritidis	*Salmonella* Enteritidis
SH	Sulfhydryl Group
SPP	Species

STEC	Shiga-toxin producing Escherichia coli
S. Typhimurium	*Salmonella* Typhimurium
TSA	Trypticase® Soy Agar
TSA + YE	Trypticase® Soy Agar plus Yeast Extract
TSB	Trypticase® Soy Broth
TSB + YE	Trypticase® Soy Broth plus Yeast Extract
USDA	United States Department of Agriculture
UV	Ultra – violet
XLD	Xylose Lysine Deoxycholate

Chapter 1: Introduction

1.1: Background

According to the Centers for Disease Control and Prevention, 48 million Americans are affected by foodborne illness each year. These occurrences lead to thousands of hospitalizations and deaths and an estimated $77.7 billion cost in the US annually (Scharff 2012) (No comma between name and year). Over the past 2 decades, the consumption of fresh produce has increased substantially due to growing consumer awareness that fruit and vegetables may result in a healthier lifestyle and prevent the occurrence of some chronic illnesses. With this increase in produce consumption, incidences of pathogen contamination can significantly affect human health. Therefore, protecting the safety of fresh produce products is a critical issue for reducing the risk of foodborne diseases.

In the produce industry, sprouts are one of the most common causes of foodborne illnesses. Typically consumed raw or lightly cooked, sprouts are found on salads, sandwiches, wraps, and in certain Asian dishes. Sprouts are known to have a very high nutritive value and consuming them may protect against the propagation of carcinogens and the development of certain chronic diseases (Wu and others 2004). Despite the nutritional advantages associated with this type of vegetable, raw or lightly cooked sprouts have been the cause of over 30 outbreaks within the United States over the last decade, resulting in over 1,800 reported illnesses (Belabre and others 2015).

Sprouts carry such a serious risk of foodborne illness because of their optimal condition for microbial growth. Seeds for sprouting require a warm and humid environment for germination and further development. The conditions required for sprouting coupled with their

neutral pH and the high bioavailability of nutrients are also ideal for microbial growth (Jung and others, 2009). Therefore, sprouts are classified as a potentially hazardous food by the United States Food and Drug Administration (FDA 2000).

Currently, the FDA recommends soaking seeds in 20,000 ppm of calcium hypochlorite to eliminate pathogens (Montville and Schaffner 2004). Even still, published data from the FDA states that "there is no single treatment that has been shown to completely eliminate pathogens on seeds or sprouts that cause foodborne illness without affecting germination or yield." Furthermore, recent sprout related foodborne illness outbreaks and published data (Fransisca and others 2011) discredit the efficacy of this government endorsed sanitation method.

Current and recently studied sprout decontamination methods include chemical disinfection, physical inactivation, and biological inhibition. These strategies have been shown to aid in bacterial reductions, however the techniques have negative attributes, some physical and others economical, which make the industry reluctant to adopt the practices. The use of 1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone (MC), an extremely stable monochlorinated *N* – halamine that possesses high antimicrobial activity, may serve as a plausible alternative without many of the negative side-effects. *N* – halamines are ideal antimicrobial agents due to their high stability in aqueous solutions and during dry storage, low cost, minimum corrosiveness in comparison to sodium hypochlorite, low toxicity to humans, and environmentally friendly nature (Demir and others 2015; Hui and Debiemme-Chouvy 2013; Kenawy and others 2007). *N* – halamines have also been tested and proven effective for various applications such as water purification systems (Chen and others 2003), food packaging and food storage materials (Quintavalla and Vicini 2002), and as antimicrobial coatings on textile, plastic, wood, glass, metal, and rubber. Specific to MC, Elder and Reed (1993) found the compound to be an excellent surface decontaminate on

radish seeds while affecting neither germination rate nor overall growth. Most recently, MC coated fabrics were found to exhibit high antimicrobial activity, resulting in 6 log reduction of *Staphylococcus aureus* within 5 minute contact time and 4 log reduction of *Escherichia coli* O157:H7 (Demir and others 2015). In summary, the above-mentioned illustrates the viability of MC compounds as a potential antimicrobial suitable for several components within a food production system.

1.2: Purpose of Study

The purpose of this study is to evaluate the antimicrobial activity of MC and its effectiveness at reducing foodborne pathogens on alfalfa sprouts. To achieve this goal, several specific objectives are listed: 1) to evaluate if MC affects the germination rate of alfalfa seeds; 2) to investigate the bactericidal activity of MC compounds toward the three primary foodborne pathogens of concern in the sprouting industry: *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes*; 3) to evaluate the efficacy of MC at reducing foodborne pathogens on inoculated sprouts; 4) to determine if MC influences the shelf life of refrigerated alfalfa sprouts; 5) to assess the quality of water used to grow alfalfa sprouts treated with MC versus alfalfa sprouts treated with city water.

1.3: Significance of Study

With the continuing occurrence of foodborne diseases and the increasing consumption of raw produce, it is important to protect fresh fruits and vegetables from being contaminated. Even though they possess a high nutritive value, raw sprouts in particular are consistently found as the source of produce related foodborne illness outbreaks. Research and past experiences have shown that intervention methods currently used in the sprout industry are oftentimes ineffective.

Even further, possible alternatives that have shown to reduce microbial loads negatively affect the physical or economical attributes of the product. Therefore, finding a novel antimicrobial agent without these disadvantages is important to not only the food industry, but also the public's health. Assessing the antimicrobial activity of MC on alfalfa sprouts has the potential to provide the fresh produce industry with an alternative for guaranteeing consumers a safe, wholesome, and affordable product.

Chapter 2: Literature Review

2.1: Food Safety and Fresh Produce

According to the Centers for Disease Control and Prevention (FDA 2015[a]), 48 million Americans are affected by foodborne illness each year. These occurrences lead to approximately 128,000 hospitalizations and 3,000 deaths. More so, according to the cost-of-illness model proposed by Robert L. Scharff (2012), the financial toll of food safety outbreaks in the United States is estimated to be $77.7 billion, annually.

Over the past 2 decades, the consumption of fresh produce has increased substantially because of growing consumer awareness that fruits and vegetables provide necessary and essential vitamins, nutrients, and fiber; all of which are components that have been found to aid in a healthy lifestyle and prevent the occurrence of some chronic illnesses (Olaimat and Holley 2012). Unfortunately, alongside this increase in fresh produce intake, the United States is also experiencing an increase in the occurrence of foodborne illnesses that stem from fresh produce sources (Olaimat and Holley 2012). Between the years of 1996 and 2010, approximately 131 produce related food safety outbreaks transpired that resulted in over 14,000 illness, more than 1,300 hospitalizations, and at least 34 deaths (FDA 2015[a]). When discussing produce related food safety incidents, contamination of foodborne pathogens can occur at any point in the food production chain. Specific stages of concern include growing, harvesting, manufacturing, processing, packing, storage, transportation, retailing, and in-home handling (FDA 2015[a]; Olaimat and Holley 2012). In a sense, every step of the farm to fork continuum poses some risk of contamination.

Along with the vulnerability of the food production system, fresh fruits and vegetables tend to receive minimal processing and are typically eaten raw (Olaimat and Holley 2012). Because of this, there is a higher probability of pathogen ingestion, especially if an organism is capable of surviving on the surface or is internalized within the produce before reaching the consumer. *Salmonell*a spp. and *Escherichia coli* O157:H7 are the two pathogens most commonly associated with produce related food safety outbreaks (Ding and others 2013; Olaimat and Holley 2012). Since 1990, sprouts in particular have been increasingly implicated in foodborne illnesses, worldwide.

2.2: Background on Sprouts

Typically consumed raw or lightly cooked, sprouts are commonly found on salads, sandwiches, wraps, and in certain Asian dishes. Sprouts of all types are known to have a very high nutritive value, serving as an excellent source of antioxidants, phenolic compounds, essential amino acids, vitamins, and minerals; biological compounds which have been seen to protect against the propagation of carcinogens and the development of certain chronic diseases (Wu and others 2004). Despite the nutritional advantages associated with this type of vegetable, sprouts are one of the most common causes of produce related foodborne illnesses. Since 1996, raw or lightly cooked sprouts have been the cause of over 30 outbreaks within the United States, resulting in over 1,800 reported illnesses (Belabre and others 2015).

In order of frequency, the type of sprout most commonly implicated in a foodborne outbreak include alfalfa, mung bean, and clover (Foodborne Illness Outbreak Database 2016). On numerous, separate occasions, *Salmonella* spp., *Escherichia coli*, and *Listeria monocytogenes* have been constantly isolated as the pathogens responsible for a majority of sprout related foodborne outbreaks (Foodborne Illness Outbreak Database 2016; Jung and others 2009).

Enteritidis, Newport, Typhimurium, Saintpaul, and Stanley are the most commonly isolated *Salmonella* serotypes in sprouts. O157:NM and O157:H7 are typical *E. coli* isolates seen in sprout related foodborne illness outbreaks (CDC 2015[a]; FDA 2015[b]).

2.3: Sprout Related Foodborne Outbreaks

Sweetwater Farms of Inman, Kansas and Jack and the Green Sprouts of River Falls, Wisconsin were identified as the sources of the two most recent sprout related foodborne illness outbreaks in the United States (CDC 2016[a]; CDC 2016[b]). Between December 1st, 2015 and January 21st, 2016, a total of 26 people, 8 of whom were hospitalized, became ill after consuming sprouts originating from Sweetwater Farms. Test results indicated that *Salmonella* Muenchen was responsible for the illnesses. Samples of irrigation water and alfalfa sprouts collected from the facility tested positive for *Salmonella*. The facility issued a recall of their products and the Kansas Department of Health and Environment issued a press release warning retailers not to sell or serve alfalfa sprouts originating from Sweetwater Farms. Eventually, further investigations revealed that the contamination originated from a tainted lot of alfalfa seeds, produced by an unnamed supplier.

Between January 17th, 2016 and February 8th, 2016, 9 people developed symptoms normally associated with an *E. coli* O157:H7 intoxication. Collaborative efforts revealed that all the patients consumed products containing sprouts that originated from Jack and the Green Sprouts. On February 24th, 2016, the Minnesota Department of Health issued a press release advising against the consumption of sprouts produced in the facility. Before conclusive microbiological tests verified the source of the outbreak, Jack and the Green Sprouts issued a voluntary recall on all of its alfalfa sprout and alfalfa onion sprout products.

During the summer of 2014, mung bean sprouts produced by Wholesome Soy Products Inc. were found to be the cause of a *Listeria monocytogenes* outbreak that resulted in five hospitalizations and two deaths. Following a 2 week break in production to clean and sanitize the facility, the company resumed normal operations. However, follow – up investigations from the FDA continuously revealed the presence of *L. monocytogenes* in irrigation water and on the mung bean sprouts. On November 7th, 2014, the company permanently stopped operating due to the inability to successfully remove the pathogen from the facility.

In 2011, Germany served as the epicenter to the largest and deadliest international sprout outbreak, to date. From May to July of that year, there were thousands of reports of bloody diarrhea, as well as an unusually high frequency of diagnoses of hemolytic uremic syndrome (HUS). According to the United States Department of Agriculture (2015), HUS is a complication related to *E. coli* infections that results in the destruction of red blood cells within the body that leads to kidney damage and sometimes death. Eventually, it was discovered that raw, mixed sprouts grown on a farm in Northern Germany were the likely cause of the outbreak. Contaminated with a highly virulent and antibiotic resistant strain of *E. coli* O104:H4, the sprouts sickened thousands and caused the death of 53 individuals (Karch and others 2012). Following further investigations and trace back studies, fenugreek seeds imported from Egypt were found to be responsible for the contaminated sprouts that were grown in Germany.

The close proximity of the two most recent sprout related foodborne outbreaks coupled with the high mortality rate of the latter relatively recent occurrence further illustrate the elevated microbial risk that is associated with raw sprouts, worldwide.

2.4: Sprouts and Food Safety

Sprouts carry such a serious risk of foodborne illness because of their optimal condition for microbial growth. Seeds for sprouting require a warm and humid environment for germination and further development. However, the conditions required for sprouting coupled with the neutral pH and the high bioavailability of nutrients are also ideal for microbial growth (Jung and others 2009). Therefore, sprouts are classified as a potentially hazardous food by the United States Food and Drug Administration (FDA 2015[b]).

Seeds are usually the primary source of contamination in sprout-associated outbreaks. The sources of seed contamination are countless. In the field, insects, birds, and wild and domestic animals alike, can easily taint the seeds. Irrigation water has also been shown to be a potential source of contamination. Water from wells, lakes, and streams used for irrigation has the potential to contain pathogens. In addition, if a livestock farm is near a sprouting facility, runoff from the farm can be a probable hazard source. Improper worker hygiene is also a likely source of sprout seed contamination (FDA 2015[b]; FDA 2004).

Once contaminated, there is some difficulty involved in decontaminating seeds. In addition, a single contaminated seed has the potential to cross contaminate the whole lot, leading to further devastation. The practices and conditions of the sprouting facility can also impact the safety of the final product (FDA 2015[b]). Besides the risks associated with seeds, poorly sanitized sprouting equipment is the second most common cause of contaminated sprouts.

2.5: *Salmonella* spp.

Salmonella belongs to the Enterobacteriaceae family and is a gram negative, facultatively anaerobic, and rod shaped bacteria. There are more than 2,500 serotypes of *Salmonella* and these serotypes are based on differences in the lipopolysaccharide (O), flagellar (H), and capsular (Vi)

antigens of the microorganism (Pui and others 2011). *Salmonella* Enteritidis, *Salmonella* Typhimurium, and *Salmonella* Heidelberg are the three most common serotypes isolated from humans (Pui and others 2011). *Salmonella* has been found in cold and warm blooded animals, as well as within the environment. Not only does this reflect the pathogen's ability to adapt to many environments, but also illustrates its success as one of the most prevalent zoonotic agents for humans. Depending on the strain, the microorganism can cause illnesses such as typhoid fever, paratyphoid fever, and food poisoning. In fact, *Salmonella* spp. are one of the leading causes of foodborne illnesses in the United States, resulting in an estimated 1.4 million infections, more than 16,000 hospitalizations, and nearly 600 deaths each year (CDC 2015[b]; FDA 2015[c]).

Salmonellosis is the infection caused by *Salmonella* spp. Once infected, victims may experience vomiting, diarrhea, fever, and abdominal cramps. The symptoms of salmonellosis tend to last from 3 to 7 days and it is typical for people to recover without the need of medical treatment. An infection stemming from *Salmonella* can easily result in the hospitalization of immunocompromised individuals. Potential food sources of *Salmonella* include eggs, poultry, meat, unpasteurized juices or milk, raw fruits and vegetables, spices, and nuts. *Salmonella* has also been isolated from some animals and their environments including, reptiles, amphibians, and birds. According to the FDA (2015[c]) and the Centers for Disease Control and Prevention (CDC 2015[b]), the most effective method of destroying the pathogen is via cooking or pasteurization.

Salmonella grows at a broad range of temperatures, from as low as 7 °C to as high as 48 °C. Highly adaptable, the pathogen can survive in pH environments ranging from 3.7 – 9.5, with the optimal pH of 6.5 – 7.5 (Pui and others 2011).

2.6: *Escherichia coli*

Escherichia coli is a gram-negative, rod-shaped, non-spore forming, facultatively anaerobic bacterium belonging to the Enterobacteriaceae family. Ubiquitous in the environment, foods, and the intestines of humans and animals, most strains of *E. coli* are harmless, but some, known as pathogenic *E. coli*, can cause diarrhea and other illnesses. Classified based on their unique virulence factors, examples of the pathogenic strains of the organism include Enterohemorrhagic *E. coli* (EHEC), Enteroinvasive *E. coli* (EIEC), Enteropathogenic *E. coli* (EPEC), and Enterotoxigenic *E. coli* (ETEC). One of the most recognized virulent strain is EHEC, also known as the Shiga-toxin producing *E. coli* (STEC). This strain includes *E. coli* O157:H7 as well as other non-O157:H7 Shiga-toxin producing *E. coli*, collectively referred to as the Big 6. The Big 6 are considered adulterants in ground beef (USDA 2015) and include the *E. coli* serogroups O26, O103, O45, O111, O121, and O145.

Unlike most disease causing bacteria, adverse health conditions can arise from very few Enterohemorrhagic *E. coli* cells. According to the FDA (2014), the infectious dose for *E. coli* O157:H7 is 10 – 100 cells. Fecal-oral transmission is the major route of transmission for the pathogenic strains of the organism. Typically lasting for 2 – 9 days, symptoms of an Enterohemorrhagic *E. coli* intoxication include severe abdominal pain, vomiting, and profuse bloody diarrhea. In extreme situations, those individuals who may be immunocompromised, such as the very young, the elderly, or the chronically ill, can experience hemolytic uremic syndrome which is characterized by bloody diarrhea, kidney disease, kidney failure, seizures, coma, and in the worst of cases, death.

Sources of pathogenic *E. coli* include animals and their environment, the feces of infected people, untreated drinking water, contaminated swimming water, and foods such as undercooked

ground beef, raw milk, and unpasteurized juices. As it was previously mentioned, the largest and the deadliest *Escherichia coli* outbreak to date originated from sprouts contaminated with *E. coli* O104:H4. Produced in Germany, the consumption of the sprouts affected almost 4,000 people and resulted in 53 deaths.

2.7: *Listeria monocytogenes*

Listeria monocytogenes is a gram-positive, rod-shaped, and facultatively anaerobic bacterium. The organism has a characteristic tumbling motility which can be attributed to its peritrichous flagella. *L. monocytogenes* is responsible for an estimated 1,600 illnesses and 260 deaths in the United States, annually (CDC 2015[c]). Concerning *Listeria*, the most susceptible and commonly infected populations include the elderly, pregnant women, newborn infants, and immunocompromised people. *L. monocytogenes* is one of the most virulent pathogens, possessing a mortality rate of 20 – 25%.

The pathogen is omnipresent in the environment; commonly found in soil, water, dust, and plant material. Unlike other pathogens, *L. monocytogenes* can grow in cool, damp environments and is capable of surviving freezing temperatures. In addition to this, the organism also exhibits heat and salt tolerance (CDC 2015[c]). Ready-To-Eat (RTE) products are of particular concern in *L. monocytogenes* contamination because they may support the growth of the pathogen during refrigerated storage. In addition, RTE products are often eaten without further cooking so there is an even greater likelihood of foodborne illness from these products if they became contaminated during processing. In a processing plant, *Listeria* can easily survive within niches such as drains, floors, and standing water (USDA 2016[a]). Therefore, it is of the upmost importance that proper sanitation and employee hygiene are achieved and maintained as to prevent cross contaminations.

The Food Safety Inspection Service (FSIS) maintains a zero tolerance policy for the presence of *Listeria monocytogenes* in Ready-To-Eat foods (USDA 2016[a]). The policy is necessary because (1) RTE foods continue to be contaminated by the pathogens and individuals continue to become ill from the consumption of the contaminated products, (2) contaminated RTE products have been shown to cross-contaminate other RTE products at the retail level, thus increasing the risk of illness, and finally (3) the infectious dose of the pathogen is thought to be low for highly susceptible populations (USDA 2016[a]). Under this rule, RTE products are considered adulterated if they contain *L. monocytogenes* or come in direct contact with a food contact surface that is contaminated with the pathogen.

Listeriosis is the infection that can develop from consuming food contaminated with *L. monocytogenes* (CDC 2015[c]). The symptoms of a listeriosis infection vary depending on the age and health conditions of the host. An immunocompetent person may suffer mild or no symptoms at all. If symptoms do develop, common ailments associated with the infection range from headache, fever, nausea, and vomiting to stiff neck, muscle aches, confusion, and loss of balance. Pregnant women are especially vulnerable to listeriosis. Complications from the infection can lead to miscarriage, stillbirth, premature delivery, or a life-threatening infection of the newborn. According to the CDC (2015[c]), 25% of *L. monocytogenes* infections in pregnant women will lead to miscarriage or stillbirth. Older adults stricken with the infection typically develop meningitis and septicemia.

Human infections involving *L. monocytogenes* outbreaks usually originate from undercooked meats and vegetables, unpasteurized milk and cheeses, and cooked or processed foods such as RTE meats and smoked seafood. In 2011, the Unites States experienced the largest *L. monocytogenes* outbreak in its history. The outbreak was associated with the consumption of

cantaloupe from a single farm and resulted in 147 illnesses, 33 deaths, and 1 miscarriage (CDC 2012).

2.8: Currently Accepted Preventive Methods

In 1997, the FDA called on the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) to (1) review current literature on sprout related food outbreaks, (2) identify organisms and production practices that pose the greatest threat to the general public's health, and (3) provide recommendations on intervention and prevention strategies (FDA 2015[b]). Established in 1988 and composed of personnel representing the government, industry, academia, and the non – profit sector, NACMCF provides unbiased, scientific advice to federal agencies (USDA 2016[b]). From their investigation, NACMCF presented the FDA with several suggestions to improve the overall quality and safety of products produced from sprouting facilities within the United States. In summary, NACMCF saw the need for enhancing food safety education for all parties including, seed producers, seed processors, seed distributors, sprout producers, sprout retailers, and consumers. In addition, the organization recommended that seeds for sprout production are grown under conditions that minimize exposure to foodborne pathogenic bacteria. The development and implementation of seed cleaning, storage, and handling practices that minimize contamination was a high priority at the time; as well as the need for sprout production facilities to possess an optimal standard of overall microbiological safety with the help of Good Manufacturing Practices (GMPs), up-to-date recall protocols, and Hazard Analysis Critical Control Points (HACCP) plans. Furthermore, NACMCF advised that seeds for sprouting be treated with one or more sanitation methods that resulted in a significant reduction of the pathogenic bacteria. Lastly, the organization urged the government and the industry to fund research related to the microbiological safety of sprouted seeds (FDA 2015[b]).

Almost two decades later, however, there has been little follow-up on several of the recommendations from NACMCF. This fact is further illustrated by the recent and steadily occurring incidents of sprout – related outbreaks. According to the FDA (2015[b]) and other data published by Montville and Schaffner (2003), "there is no single treatment that has been shown to completely eliminate pathogens on seeds or sprouts that cause foodborne illness without affecting germination or yield." Until an appropriate disinfection treatment is discovered, the FDA will continue to recommend soaking seeds in 20,000 ppm of chlorine solution, in the form of calcium hypochlorite, to eliminate pathogens (Montville and Schaffner 2003). Alternatively, in lieu of the aforementioned recommendation, the FDA permits sprouting facilities to use any other sanitation method or combination of sanitation methods that result in a demonstrated 5-log bacterial reduction (FDA 2015[b]; Montville and Schaffner 2003).

Several sprout-related foodborne outbreaks question the effectiveness of the FDA's recommendation of 20,000 ppm of chlorine for seed treatment. A number of seed producers previously implicated in a food safety outbreak claim to have followed the FDA's sanitation guidelines. Even so, the facility still fell victim to national headlines exposing their contaminated product. Furthermore, published results have consistently shown that this method was ineffective at eliminating pathogens that were inoculated in the laboratory setting (Fransisca and others 2011). Even with the availability of published data that discredit this sanitation method, the FDA currently supports the efficacy of 20,000 ppm of chlorine to reduce the potential of sprout-related foodborne illness outbreaks.

2.9: Potential Preventive Methods

Decontamination steps are generally geared to un-sprouted seeds versus fully grown sprouts. This practice stems from the fact that there is usually less debris and superfluous organic matter in seeds in comparison to the sprouted product. In addition, the physical structure of seeds are generally more resistant to antimicrobial treatments than the delicate sprouts (FDA 2015[b]). Along with these factors, seeds usually have a lower microbial load ($10^2 – 10^6$ CFU/g) compared to the germinated product ($10^8 – 10^{11}$ CFU/g) (Jung and others 2009); therefore it is easier to sanitize and/or disinfect seeds. As mentioned previously, the conditions of the sprouting process are quite conducive for microbial growth. Thus, it is widely accepted that the most effective intervention step involves the treatment of seeds before exposure to bacterially favorable sprouting conditions (FDA 2015[b]). The objective is to remove as many microorganisms from the seeds to prevent or lower microbial contamination in sprouts. Potential treatments include chemical disinfection, physical inactivation, and biological inhibition (Ding and others 2013). In the sprout industry, any alternative decontamination process must not affect the germination rate, the total yield of the final product, or the organoleptic quality of neither the seeds nor the freshly sprouted produce (Jung and others 2009). In addition, when discussing new sanitation practices, the cost, treatment time, required equipment, and issues with regulatory compliance must always be considered.

Biological Control Methods

Biological interventions provide a promising option for reducing pathogenic microorganisms while decreasing or completely eliminating the use of synthetic chemicals. Bacteriocins, bacteriophages, and protective cultures have all been studied as possible strategies

of reducing the amount of pathogenic organisms seen in seeds for sprouting. Known as biopreservation techniques, the above – mentioned methods improve the stability and safety of food products without altering sensory qualities, by using certain microorganisms, their metabolites, or both (Melero and others 2012; Settanni and Corsetti 2008).

Bacteriocins are ribosomally synthesized, low molecular mass proteins produced by bacteria that can kill or inhibit the growth of other bacteria. The antimicrobial toxins produced by these organisms are very specific, usually only proving effective against microorganisms within the same species or a closely related species (Settanni and Corsetti 2008). In the sprout industry, an ideal antimicrobial agent should have the capability to inhibit a wide variety of microorganisms, including foodborne pathogens. However, if bacteriocins are used, several microbial strains need to be added for proper biological control. In addition, several studies found that, in general, bacteriocins are active against gram positive bacteria and not gram negative bacteria (Cobo Molinos and others 2008; Bari and others 2005; Cobo Molinos and others 2005). Pathogens of concern include both gram positive and gram negative species, indicating the use of bacteriocins in the sprout industry as inefficient.

Bacteriophages are viruses that infect and kill bacterial cells. Non-threatening to humans, bacteriophages colonize bacterial cells, reproduce within their hosts, and ultimately lyse the cells, killing the organisms. When studied in sprout systems, bacteriophages resulted in minimal bacterial reductions (0.5 to 3 log reduction) (Kocharunchitt and others 2009; Pao and others 2004). The binding of the phages on the seeds was reported as an issue, limiting the effectiveness of this biological treatment at decreasing pathogens on the sprouts (Pao and others 2004).

Protective cultures are bacteria especially selected and developed for their ability to control the growth of pathogenic and spoilage microorganisms in foods. In general, the

organisms within a protective culture are chosen because of their inhibitory effect against other microorganisms based on their competition for nutrients, their production of antagonistic compounds such as organic acids, bacteriocins, enzymes, and/or hydrogen peroxide, or a combination both characteristics (Melero and others 2012). Under proper environmental conditions, the use of protective cultures in sprouts has shown promising results at decreasing the microbial load while not affecting the sprout yield (Ye and others 2010). In unfavorable conditions, however, protective cultures have been unsuccessful at inhibiting pathogens. The environment for sprout production has been shown to restrict the growth of protective strains, ultimately limiting the culture's inhibitory effect (Cobo Molinos and others 2008; Bennik and others 1999).

Chemical Control Methods

The FDA's current recommendation of using 20,000 ppm of calcium hypochlorite to sanitize seeds for spouting is considered as a chemical intervention. As mentioned previously, the effectiveness of this treatment is under much debate. In addition, a further limitation of the calcium hypochlorite treatment includes the possible hazards associated with producing, transporting, and handling large amounts of the chemical (Sikin and others 2013). Alternative chemical treatments currently being studied include organic acids, ozone, electrolyzed water, and chlorine dioxide.

Predominantly justified by its higher antimicrobial efficacy in comparison to chlorine, chlorine dioxide (ClO_2) is typically used in place of chlorine-based sanitizers primarily because of its higher oxidation capacity. In addition, ClO_2 is not as sensitive to changes in pH and the presence of organic matter, like chlorine is, and does not produce as many potentially

carcinogenic byproducts (Roberts and Reymond 1994). On the downside, studies have shown that when acting alone, chlorine dioxide is not very effective against pathogens, leading to microbial reduction values similar to that of chlorine (Kim and others 2010). Furthermore, products treated with ClO_2 gas have been shown to contain high levels of chloride and chlorite byproducts, which typically pose major regulatory issues (Trinetta and others 2011).

The use of ozone has also been studied as an option for sanitizing seeds for sprouting. Microbial inactivation is attained by the reactive and unstable hydroxyl radicals associated with the chemical. In relation to sprouts, ozonized water has been the most studied, however, several studies found microbial reductions to be minimal (Khadre and Yousef 2001; Cherry 1999; Kim and others 1999). This issue is accredited to the fact that ozone quickly dissociates back to oxygen in water, reducing the antimicrobial effects (Sikin and others 2013). In addition to the lack of bactericidal activity, sensory panels found negative organoleptic characteristics associated with sprouts treated with ozone when compared to those that were not (Wade and others 2003).

Organic acids, such as lactic and acetic acids, are desirable because they are environmentally friendly, cost-effective, proven as antibacterial agents, and are generally recognized as safe (GRAS) by the FDA. Disadvantages do coincide with the use of these chemicals. When given to sensory panelists, some studies found that the use of certain organic acids had a negative effect on the flavor quality of the sprouts (Singla and others 2011). In addition, most studies illustrated that achieving significant microbial reductions required an extended contact time between the acid and the seeds (Pao and others 2008). Which is, for the sprouting industry, an impractical requirement.

Emerging as a new antimicrobial agent, electrolyzed water is being studied for its potential use in food safety. The electrolyzed water system employs three mechanisms of antimicrobial activity: pH, free chlorine, and oxidation reduction potential (Hati and others 2012). Individually, each of these components have been shown to possess antimicrobial activity. So it was speculated that the combination of all three would have at least an additive effect, if not synergistic. When applied to the system of seeds for sprouting, however, the electrolyzed water did not perform as well as hoped. In several studies, the novel disinfectant was only able to achieve minimal bacterial reductions (< 2 log CFU/g of pathogens) (Kim and others 2003). In addition to its ineffectiveness at seed decontamination, the application of this type of intervention is limited because the release of chlorine from the electrolyzed water system can have adverse effects on the environment, human health, and potentially lead to the corrosion of equipment, which can prove costly (Sikin and others 2013).

Several of the previously discussed chemical interventions have been shown to be more effective when combined with one or more alternative treatments, especially physical decontamination methods such as thermal inactivation, irradiation, and high pressure.

Physical Control Methods

The biological and chemical interventions listed above have been limited to only surface disinfection of the sprout seeds. Physical disinfection methods have been proven to possess higher penetration power, thus aiding in their ability to reach and inactivate internalized microorganisms (Sikin and others 2013). In addition to the ability of these techniques to reach sheltered pathogens, physical intervention methods in the fresh produce industry are usually

associated with a healthier and more natural image, especially when compared to chemical techniques.

Heat treatments, both dry and wet, are considered inexpensive and simple methods for seed decontamination. In general, when applied to seeds, there is usually greater than a 5 log CFU/g reduction (Weiss and Hammes 2005). Problems do arise if the temperature is too high or the treatment time is too long. An improper combination of temperature and time can lead to a decline in germination rate and sprouts with lower textural qualities and nutritional value (Farhangi and Valadon 2006).

Irradiation is another type of physical treatment typically derived from the radioactive isotopes cobalt-60 (^{60}Co) or cesium-137 (^{137}Cs). In the United States, the FDA (2004) has approved the use of radiation on sprout seeds up to a maximum of 8 kilograys. Several researchers have reported large microbial reduction (≥5 log CFU/g) by using radiation dosages between 1 and 5 kGy (Kim and others 2006; Bari and others 2004). Even with the existence of published data that illustrates considerable inactivation levels in comparison to other decontamination methods, irradiation is seldom used in the sprout industry. Studies have shown that irradiation treatments have adverse effects on seed germination and overall quality of the sprouted product (Yun and others 2013; Kim and others 2006). In addition, the equipment for seed irradiation is costly. Lastly consumers do not fully accept irradiated foods. Overall, it is not economically attractive for the sprout industry to adopt the practice of irradiating sprout seeds.

Ultra-violet light has also been researched as another option in seed decontamination. The intervention method exerts its bactericidal effect by creating pyrimidine-base dimers and DNA-protein crosslinks to decrease cell vitality and result in cell death (Blatchley and Peel 2001). UV radiation in the sprout industry has several benefits. The process does not leave

residues on food products, has no government regulation on the amount of usage, and requires little safety equipment when it comes to worker well-being (Yaun and others 2004). Its effectiveness is restricted to surface decontamination, however, because of the poor penetration capability of UV – light. Similar to the intervention strategies previously discussed, UV radiation is considered more effective in the decontamination of sprouting seeds when applied in conjunction with another antimicrobial or bactericidal method.

High-pressure decontamination techniques have been proven effective for eliminating pathogenic microorganisms in seeds. The efficacy of this intervention strategy is thought to be the instantaneous and uniform action of pressure throughout the food sample, regardless of size and shape (Farkas and Hoover 2000). Even with conclusive data on the antimicrobial success of high pressure treatments, the sprout industry has been slow to adopt the practice. Evidences of delayed germination and decreases in germination rates eliminate the potential use of high pressure in the sprout industry (Ariefdjohan and others 2004; Farkas and Hoover 2000).

The previously discussed intervention strategies all have been shown to ascertain a degree of bacterial reduction in seeds for sprouting. However, all the techniques have negative attributes, some physical and others economical, which make the industry reluctant to adopt these aforementioned practices. In this research, the use of MC (1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone) may serve as a plausible alternative to the previously suggested decontamination methods. The MC is an extremely stable monochlorinated *N* – halamine that possesses high antimicrobial activity, without many of the negative side-effects.

2.10: *N* – halamines

N – halamines are compounds containing one or more nitrogen – halogen covalent bonds, typically formed by the halogenation of imide, amide, or amine groups (Hui and Debiemme-

Chouvy 2013). Gaining noticeable recognition in the past decade, these types of compounds display significant antimicrobial activity. Superior biocidal function has been seen in a broad range of microorganisms including gram positive and gram negative bacteria (Demir and others 2015; Li and others 2014), yeasts, fungi, and viruses (Demir and others 2015; Sun and others 1994). Furthermore, in the lab setting, some microorganisms were inactivated in a contact time of less than 5 minutes and others experienced as much as a 9 log bacterial reduction during extended contact times. (Li and others 2014).

N – halamines exert their powerful biocidal activity due to the oxidation state of halogen atoms in the N – Cl groups. Research has indicated that *N* – halamines are able to dissociate and become free halogens in aqueous solutions to terminate targeted pathogens or oxidize microbial membranes (Kenawy and others 2007). Beyond this however, the exact mode of action of the antimicrobial compound is not confirmed. There are three hypothesized modes of action involving the inactivation of microorganisms when they come in contact with *N* – halamines. The first theory suggests that the Cl group on the *N* – halamine absorbs into the membrane surface of the microorganism, causing cell lysis by interacting with the phospholipid bilayer. This action leads to the leakage of ions and other functional intercellular constituents to the outside of the cell, ultimately killing the microorganism (Ikeda and others 1984). Others hypothesize that the *N* – halamine may oxidize the proteins on the membrane of the microorganism by reacting with the –SH groups of the proteins (Siedenbiedel and Tiller 2012). This action disrupts the disulfide structure, compromises the integrity of the cellular membrane, leads to cellular leakage, and ultimately results in cell death. Lastly, others believe that upon contact with the microorganism, the oxidative halogen constituent of the *N* – halamine decomposes and diffuses into the microbial cell through the cell membrane. This action disrupts

the ion balance of the cell and ultimately prevents the synthesis of several essential proteins, resulting in cell death (Demir and others 2015).

In addition to their superior antimicrobial activity, *N* – halamines are ideal antimicrobial agents due to their high stability in aqueous solutions and during dry storage, low cost, minimum corrosiveness in comparison to sodium hypochlorite, low toxicity to humans, and environmentally friendly nature (Demir and others 2015; Hui and Debiemme-Chouvy 2013; Kenawy and others 2007). Considering these advantageous characteristics, *N* – halamines have been tested and proven effective for various applications such as water purification systems (Chen and others 2003), food packaging and food storage materials (Quintavalla and Vicini 2002), and as antimicrobial coatings on textile, plastic, wood, glass, metal, and rubber.

A unique characteristic that illustrates the value of an *N* – halamine as an antimicrobial agent involves its rechargeable nature. Even after disinfection, when the halogens are consumed in the antimicrobial reactions, *N* – halamines are easily reactivated. By simply reintroducing the *N* – halamine to a Cl or Br donor solution such as sodium hypochlorite or sodium hypobromite, the biocidal agent is re-charged and capable of further antimicrobial reactions (Hui and Debiemme-Chouvy 2013).

2.11: MC (1-chloro-2,2,5,5-tetramethyl-4-imidazolidinone)

MC, 1 – chloro-2,2,5,5-tetramethyl-4-imidazolidinone, is a type of monochlorinated *N* – halamine which possesses high stability and has demonstrated superior antimicrobial activity in several diverse applications. Most recently, researchers found that MC coated fabrics exhibited high antimicrobial activity, resulting in 6 log reduction of *Staphylococcus aureus* within 5 minutes contact time and 4 log reduction of *E. coli* O157:H7 (Demir and others 2015). The same study also evaluated the viability of the MC coated fabrics over an extended period of time.

When stored in dark environments, the fabrics maintained their initial chlorine content for 6 months (Demir and others 2015), this further supporting the abovementioned MC stability claim.

In 2013, U.S. Patent Number 8,563,587 B2 (Bridges and others 2013) was published, claiming the validity of using MC as the primary ingredient in the treatment of aquatic diseases. Envisioned to be made available in a manner that ensures the slow release of the active components, the inventors intend to introduce MC, at a concentration of 20 ppm, to an aquatic environment for about 6 hours. This treatment is speculated to remedy aquatic diseases ranging from fin rot to fungal and bacterial infections.

Elder and Reid (1993), reported that MC was an excellent surface decontaminate when used on the seeds of radishes. In addition to the antimicrobial efficacy of the compound, the researchers also found that MC affected neither the germination rate of the seeds nor the overall growth of the radishes (Elder and Reid 1993).

Both the oral and dermal toxicity of MC has been tested on laboratory rats. The oral LD_{50} value for MC was determined to be 338 mg/kg, with signs of toxicity including hypo-activity, abnormal respiration, ocular discharge, and a decline in muscle coordination. The dermal toxicity study revealed minimal topical irritation, resulting in a dermal LD_{50} value and a no observed effect level (NOEL) of >5000 mg/kg (Parent 2000).

The above-mentioned illustrates the viability of MC compounds as a potential antimicrobial suitable for several components within a food production system.

Chapter 3: Materials and Methods

3.1: Culture preparation

Salmonella Enteritidis, Typhimurium, *Escherichia coli* O157:H7, and *Listeria monocytogenes* were used in this study. Separately, one loop of *Salmonella* Enteritidis and *Salmonella* Typhimurium from frozen culture, was added into 10 mL of Trypticase® Soy Broth (TSB) (Becton, Dickinson, and Company Sparks, MD). The cultures were incubated at 37 °C for 16 h and then transferred into another sterile tube with 10 mL of TSB. The incubation procedure was repeated three times to ensure the viability of the bacterial cultures. Each culture was then streaked on Trypticase® Soy Agar (TSA) (Becton, Dickinson, and Company Sparks, MD) and the plates were incubated in the same conditions mentioned above. An isolated colony from each plate was selected and added into 10 mL of TSB and cultures were incubated for 16 h at 37 °C. Then, 10 mL of each serotype culture were combined together into a cocktail. The *Salmonella* cocktail was centrifuged at 3,800 xg for 15 min. The supernatant was discarded and the pellet was washed twice with Butterfield's Phosphate Buffer (BPB) through centrifugation. The bacteria were re-suspended in BPB for use as a stock bacterial suspension.

One loop of *Escherichia coli* O157:H7 from frozen culture was grown in TSB for 16 h at 37 °C. One loop of *Listeria monocytogenes* from frozen culture was added into 10 mL of TSB plus 0.5% yeast extract (TSB+YE) for 16 h. The procedures used for *Escherichia coli* O157:H7 and *Listeria monocytogenes* preparation were the same as described above for *Salmonella*. The absorbances at OD_{640nm} of all three bacterial suspensions were measured for estimating the bacterial population and preparing the inoculums. The designed bacterial inoculums were prepared through dilution from stock bacterial suspensions.

Actual additional population measurement was performed by preparing a 10 – fold serial dilution up to 10^{-8} from the stock bacterial suspensions. Each dilution was plated onto selective media based on the bacterial culture (Xylose Lysine Deoxycholate Agar (XLD) (Neogen Corporation Lansing, MI) for *Salmonella*, MacConkey Agar (MAC) (Neogen Corporation Lansing, MI) for *E. coli* O157:H7, and Modified Oxford Medium (MOX) (Neogen Corporation, MI) for *L. monocytogenes*), then plates were incubated at 37 °C for 24 h. Colonies were counted to calculate the actual populations of the bacterial inoculums.

3.2: Effect of MC on Alfalfa Seed Germination Rate

In the sprouting industry, the effect of an antimicrobial on seed germination rate is a crucial factor in determining if the treatment is adopted. This study determined if MC affects the rate of germination of alfalfa seeds. Three sterile petri dishes were lined with two layers of Bounty 2 – ply paper towels and 1 layer of No. 1 filter paper (GE Whatman Pittsburg, PA). One hundred alfalfa seeds (Now Real Food: Certified Organic Alfalfa Seeds Bloomingdale, IL) were placed into each of the 3 petri dishes. MC solutions of 0, 0.01, and 0.05% were made fresh daily by dissolving the compound into distilled water. Each filled petri dish was treated with approximately 5 mL of the MC solution. The treated seeds in petri dishes were covered and placed in a 27 °C incubator for 24 h. Following incubation, the germinated seeds on each petri dish were counted and recorded. The alfalfa seeds were treated with 5 mL of MC solution again. The samples were incubated at 27 °C for an additional 24 h. After incubation, the germinated seeds on each petri dish were counted and recorded. The germination rates were evaluated in triplicate.

3.3: Microbial Loads of Untreated Alfalfa Seeds and Sprouts

The background microorganisms of *Salmonella*, *Staphylococcus*, *Listeria*, *E. coli*, and coliforms on alfalfa seeds and alfalfa sprouts were evaluated. Selective and differential media were used to isolate and identify the background microorganisms. XLD, Bismuth Sulfite Agar (BSA) (Neogen Corporation, MI), and Hektoen Enteric Agar (HE) (Neogen Corporation Lansing, MI) were used to identify *Salmonella*. Criterion™ Baird – Parker Agar (BP) (Hardy Diagnostics Santa Maria, CA) was used in the identification of *Staphylococcus* species while MOX was utilized for *Listeria* isolation. Lastly, 3M™ Petrifilm™ *E. coli*/Coliform and Aerobic count plates (3M Food Safety St. Paul, MN) were used in the enumeration of *E. coli*, coliform, and total aerobic microorganisms, respectively.

Analysis of Alfalfa Seeds Microflora

In this study, 25 g of alfalfa seeds and 225 mL of TSB were put into a filtered stomacher bag (Nasco, Ft. Atkinson,WI) and the sample was stomached (Stomacher® 400 Circulator Seward Ltd West Sussex, UK) for 2 min on a medium setting. The pulverized contents were serially diluted up to 10^{-2} and each dilution was plated on the abovementioned media in triplicate. The plates were incubated according to the manufacturer instructions. XLD, BSA, HE, BP, and MOX were incubated for 24 h at 37 °C. 3M™ Petrifilm™ *E. coli*/Coliform were incubated for 48 h at 37°C and the Aerobic count plates were incubated for 48 h at 25 °C. Following incubation, colonies were recorded for background microorganism analysis.

Analysis of Alfalfa Sprouts Microflora

Alfalfa sprouts were grown in sprouters (Tribest Freshlife 3000 Automatic Sprouter, Tribest Corporation Anaheim, CA) for 5 d at 27 °C. In this study, 25 g of alfalfa sprouts and 225 mL of

TSB were put into a filtered stomacher bag and the sample was stomached for 2 min on a medium setting. The pulverized contents were serially diluted up to 10^{-6} and each dilution was plated on the abovementioned media in triplicate. The plates were incubated according to the manufacturer instructions. Following incubation, colonies were counted for analyzing the background microorganisms of alfalfa sprouts.

3.4: Antimicrobial Efficacy of MC on Foodborne Pathogens

The concentrations of MC used in this study were 0, 0.01, and 0.05%. *Salmonella* at 1 x 10^8 CFU/mL was prepared with the same procedures mentioned above. An aliquot of 100 µL of *Salmonella* Typhimurium and Enteritidis cocktail was added to 9.9 mL of MC solution and the mixture was vortexed thoroughly. Each concentration was triplicated. At contact times of 0, 5, 10, 30, and 60 min, 100 µL of the mixture from each tube was taken and diluted decimally with peptone water to 10^{-6}. Each dilution was plated on TSA in duplicate. The plates were incubated at 37 °C for 24 h and the colonies were counted for analysis. In addition, the actual population of the inoculum was determined by the spread – plate method.

The protocol used to test the biocidal activity of MC against *L. monocytogenes* and *E. coli* O157:H7 was the same as the method used for *Salmonella*.

3.5: Antimicrobial Efficacy of MC on Microbial Inoculated Alfalfa Sprouts

Nalidixic acid resistant *E. coli* O157:H7, nalidixic acid resistant *Salmonella* cocktail of Typhimurium and Enteritidis, and streptomycin resistant *Listeria monocytogenes* were used in this study. The bacterial inoculums of 1 x 10^6 CFU/mL used in this experiment were prepared with the same procedures mentioned above. Approximately 900 g of alfalfa sprouts, 200 mL of

the bacterial suspension, and 1800 mL of BPB were combined and slowly agitated for 1 min to allow for bacterial inoculation. The alfalfa sprouts were removed from the mixture and air dried in a biosafety cabinet for 20 – 30 min for further bacterial attachment. After dried, 300 g of alfalfa sprouts were submerged in either 0, 0.01, or 0.05% MC solutions. At contact times of 0, 5, 10, 30, and 60 min, 25 g of the alfalfa sprouts were removed from each treatment, placed in a filtered stomacher bag, and 225 mL of 0.05 N sodium thiosulfate solution were added. The sodium thiosulfate was able to quench chlorine residuals, preventing further microbial killing. The samples were stomached for 2 min on a medium setting and a 10 – fold serial dilution was made from the contents of the bag up to 10^{-4}. Each dilution was plated on either TSA + 100 ppm nalidixic acid (*Salmonella* and *E. coli* O157:H7) or TSA + 50 ppm streptomycin (*Listeria monocytogenes*), in duplicate. The plates were incubated at 37 °C for 24 h and colonies were counted for analysis.

3.6: Longevity of MC's Antimicrobial Activity on Alfalfa Sprouts

The microbial quality of alfalfa sprouts treated with 0.01% and 0.05% MC was evaluated over the length of 7 d. Alfalfa sprouts were treated with 0, 0.01, or 0.05% MC solutions for 2 min. Following exposure to the MC solutions, the alfalfa sprouts were allowed to air dry in a biosafety cabinet for 20 – 30 min. Once dried, approximately 350 g of alfalfa sprouts from each treatment group were transferred into one gallon Ziploc bags and stored at 8 °C. On day 0, 1, 3, 5 and 7, 25 g of the alfalfa sprouts were removed from each treatment group, placed in a filtered stomacher bag, and 225 mL of BPB were added. The samples were stomached for 2 min on a medium setting and a 10 – fold serial dilution was made from the contents of the bag up to 10^{-6}.

Each dilution was plated on 3M™ Petrifilm™ Aerobic count plates, in duplicate. The plates were incubated at 25 °C for 48 h and colonies were counted for microbial analysis.

3.7: MC Effect on Microbial Change in Water for Sprouting

The water for sprouting alfalfa seeds was treated with 0% and 0.05% MC and total aerobic plate count microorganisms were evaluated over a period of 5 d. During the first trial, the water in the sprouter for each treatment was changed daily. Following the 5 d period, the alfalfa sprouts were harvested, the sprouters were cleaned and sanitized, and new alfalfa seeds were grown. During this trial, the water in the sprouter for each treatment was not changed over the 5 d period. From day 0 to day 5, 1 mL of solution was taken from each treatment group daily and serially diluted with BPB to 10^{-6}, in triplicate. Each dilution was plated on 3M™ Petrifilm™ Aerobic count plates, in triplicate. The plates were incubated at 25 °C for 48 h and colonies were counted and analyzed for water quality over the 5 d period.

3.8: Statistical Analysis

Statistical analysis was performed using SAS 9.4 (SAS Institute Inc., Cary, NC). Analysis of variance (ANOVA) was performed with Tukey's multiple range test with comparison of means at a 95% confidence level.

Chapter 4: Results and Discussion

4.1: MC Effect on Alfalfa Seed Germination Rate

The effect of MC on the germination rate of alfalfa seeds was evaluated over a 5 d period. There was no significant difference found among the control, the 0.01% MC treatment, and the 0.05% MC treatment. From these findings, it is assumed that MC at the tested concentrations does not affect the germination rate of alfalfa seeds (Figure 4.1.1). In addition, the alfalfa seeds germinated at a rate of greater than 90%, which is in agreement with industry specifications (USDA 2016[b]; Sikin and others 2013). Therefore, the MC treatment would not have a negative effect on this quality characteristic.

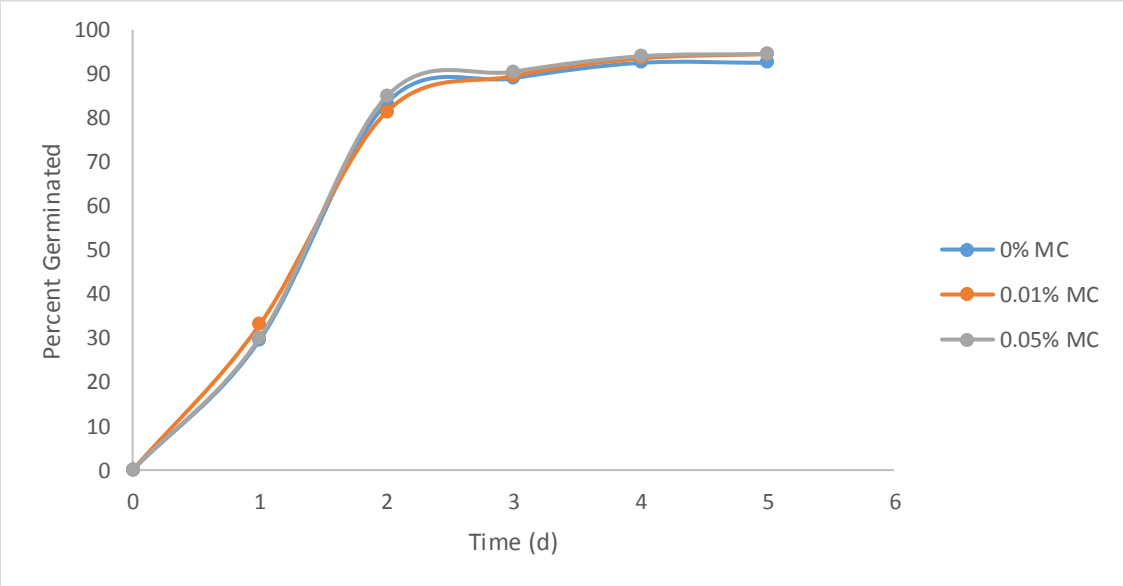

Figure 4.1.1: The effect of MC on the germination rate of alfalfa seeds

4.2: Microbial Loads of Untreated Seeds and Sprouts

The background microorganisms of *Salmonella*, *Staphylococcus aureus*, *Listeria monocytogenes*, *E. coli*, and coliforms on alfalfa seeds and alfalfa sprouts were evaluated. The microbial analysis of the alfalfa seeds carried an aerobic bacterial population of 2.11×10^3 CFU/g. These results are similar to other reported values for the microbial load of alfalfa seeds which range from $10^2 – 10^6$ CFU/g (Sikin and others 2013; Jung and others 2009). The microbial load of alfalfa seeds is variable due to differences in seed production, storage, and handling (USDA 2016[b]). The seeds carried a coliform bacterial population of 1.1×10^3 CFU/g. These numbers align with those previously reported in the literature (Sikin and others 2013; Jung and others 2009; Soylemez and others 2001). When analyzing the seeds for background microorganisms, *Salmonella*, *S. aureus*, *L. monocytogenes*, and *E. coli* were undetectable using spread plate method.

Alfalfa seeds were grown for 5 d and the background microorganisms were evaluated (Table 4.2.1). The sprouts carried an aerobic bacterial population of 1.03×10^8 CFU/g, a coliform bacterial population of 7.75×10^7 CFU/g, and a *S. aureus* bacterial population of 5.55×10^5 CFU/g. The results of the aerobic bacterial and coliform populations align with previously reported data on alfalfa sprouts, finding bacterial populations which range from $10^7 – 10^{11}$ CFU/g (Jung and others 2009; Soylemez and others 2001). Seo and others (2010) reported similar results (2.5 – 7.5 Log CFU/g) for the microbial load of *S. aureus* isolated from various species of sprouts. Generic *E. coli*, *Listeria* and *Salmonella* were not recovered at the detection limit of this experiment (Table 4.2.1).

Table 4.2.1: Microbial Load of Alfalfa Sprouts

Microorganism	Bacterial Population (CFU/g)
Total Aerobic	1.03×10^8
Coliforms	7.75×10^7
E. coli	0*
Listeria monocytogenes	0*
Salmonella	0*
Staphylococcus aureus	5.55×10^5

*Detection limit of >100 CFU/g

4.3: Antimicrobial Efficacy of MC on Foodborne Pathogens

Table 4.3.1 shows the results of the antimicrobial activity of MC against *Salmonella*. Compared with the control group, the two concentrations of MC showed strong antimicrobial activity. In general, at each contact time, with an increase in MC concentration, the log reduction of *Salmonella* significantly increased (Figure 4.3.1). MC at 0.01% concentration inactivated almost 3 logs of *Salmonella* within 5 min contact time in BPB. While at the 0.05% level, the MC was able to achieve more than a 5 log bacteria reduction. At 10 min of contact time, the bacteria reduction increased to more than 8 logs (complete kill) when the MC concentration was increased from 0% to 0.05%. Similar results were seen at 30 min of contact time. At 60 min of contact, both 0.01% and 0.05% MC concentrations were able to inactivate 100% of the inoculated bacteria.

The reduction of *Salmonella* also increased with longer contact time between the bacteria and the MC in BPB (Figure 4.3.1). The control group did not show any significant difference in *Salmonella* reduction at different contact times, however, for the MC treated groups, the biocidal activity increased with longer contact times. In the 0.01% MC solution, the log reduction from

contact times 5 min to 60 min resulted in a *Salmonella* log reduction from 2.76 at 5 min to 8.11 which was a complete kill at 60 min. At 10 min of contact time, 0.05% MC was able to completely kill all the inoculated bacteria (8.11 log).

Table 4.3.1: Log Reduction of *Salmonella* Treated with MC in BPB

MC Concentration	Contact Time (min)			
	5	**10**	**30**	**60**
0.00%	1.24 ± 0.02 ^{aA}	0.90 ± 0.27 ^{aA}	1.03 ± 0.04 ^{aA}	1.13 ± 0.07 ^{aA}
0.01%	2.76 ± 0.11 ^{bA}	3.22 ± 0.05 ^{bB}	4.95 ± 0.02 ^{bC}	8.11 ± 0.00 ^{bD}
0.05%	5.12 ± 0.17 ^{cA}	8.11 ± 0.00 ^{cB}	8.11 ± 0.00 ^{cB}	8.11 ± 0.00 ^{bB}

* Inoculum: 8.11 Log CFU/mL
a,b,c – same character means no difference within a column ($p<0.05$)
A,B,C – same character means no difference within a row ($p<0.05$)

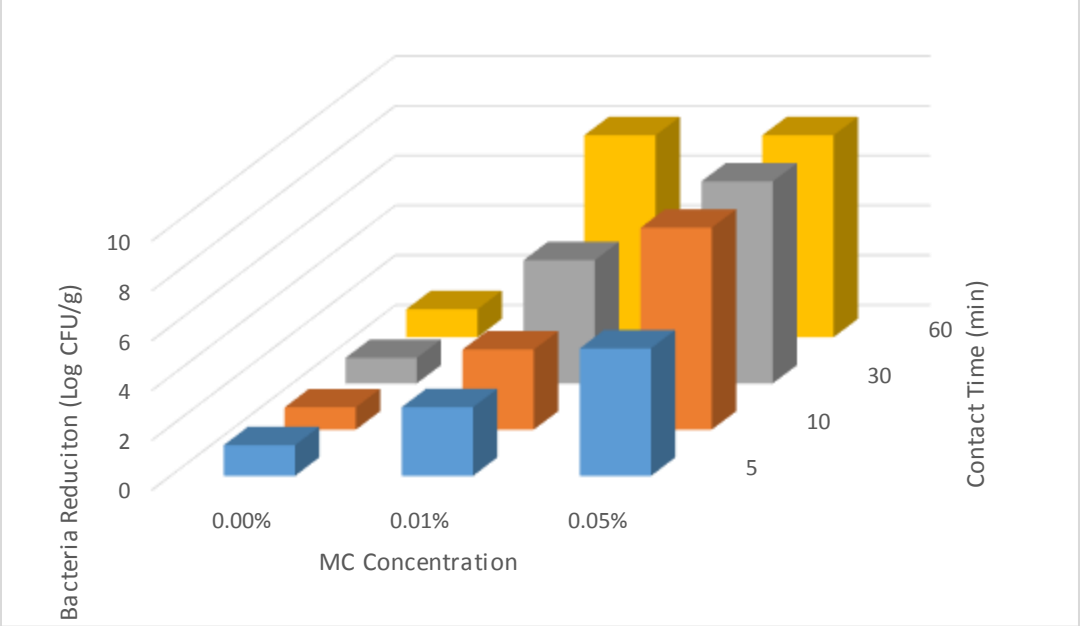

Figure 4.3.1: Antimicrobial activity of MC against *Salmonella* in BPB

Table 4.3.2 displays the results of the biocidal activity of MC against *E. coli* O157:H7. As in the *Salmonella* inactivation test, at each contact time, the population of *E. coli* O157:H7 significantly decreased as the MC concentration increased (Figure 4.3.2). At 10 min of contact time, the control group resulted in 0.94 log reduction of *E. coli* O157:H7, while the 0.01% MC solution and 0.05% MC solution resulted in 2.67 and 5.01 log reductions of the bacteria, respectively. At 30 min of contact, 0.01% MC resulted in a 4.69 log reduction of *E. coli* O157:H7, while 0.05% MC was able to inactivate 100% (8.08 log) of the inoculated bacteria. Similar trends were seen at 60 min of contact time.

The biocidal efficacy of MC increased as the contact time increased between the compound and the bacteria (Figure 4.3.2). The control group did not show any significant differences in *E. coli* O157:H7 reduction at the different contact times. The 0.01% MC concentration resulted in a 1.92 log reduction of the inoculated bacteria at 5 min, and steadily increased to a log reduction of 2.67, 4.69, and 6.41 at contact times of 10, 30, and 60 min, respectively. At 5 min contact time, the solution of 0.05% MC reduced the bacterial population by about 4 logs. The *E. coli* O157:H7 was completely inactivated when the contact time was extended to 30 min.

Table 4.3.2: Log Reduction of *E. coli* O157:H7 Treated with MC in BPB

MC	**Contact Time (min)**			
Concentration	**5**	**10**	**30**	**60**
0.00%	1.04 ± 0.06^{aA}	0.94 ± 0.06^{aA}	1.11 ± 0.14^{aA}	0.93 ± 0.11^{aA}
0.01%	1.92 ± 0.13^{bA}	2.67 ± 0.04^{bB}	4.69 ± 0.11^{bC}	6.41 ± 0.07^{bD}
0.05%	3.93 ± 0.21^{cA}	5.01 ± 0.12^{cB}	8.08 ± 0.00^{cC}	8.08 ± 0.00^{cC}

* Inoculum: 8.08 Log CFU/mL
a,b,c – same character means no difference within a column ($p<0.05$)
A,B,C – same character means no difference within a row ($p<0.05$)

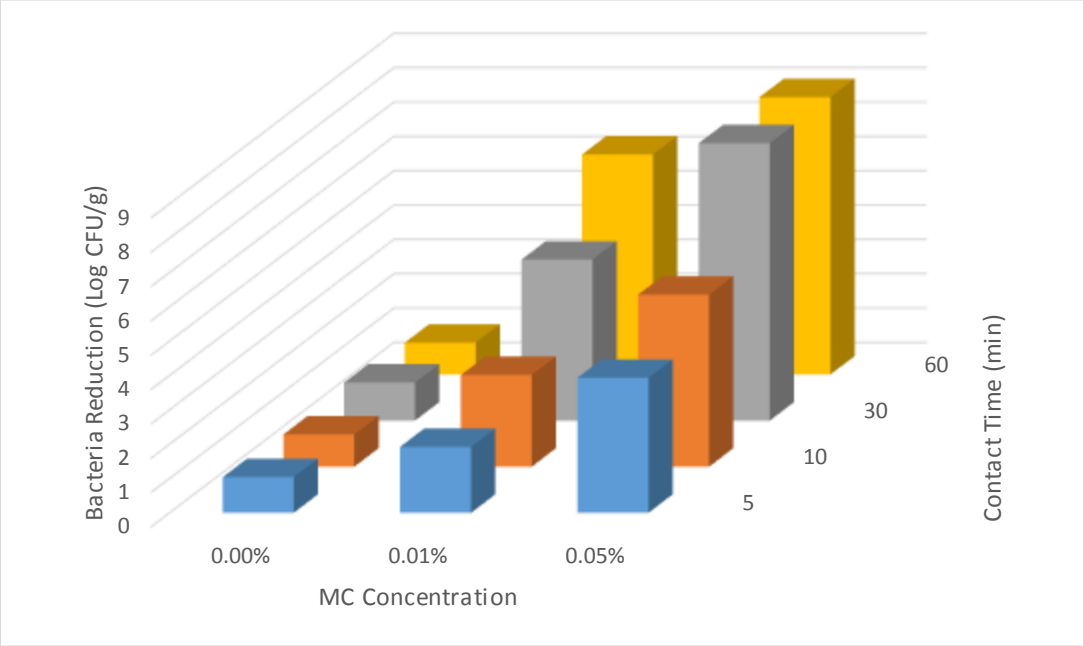

Figure 4.3.2: Antimicrobial activity of MC against *E. coli* O157:H7 in BPB

Table 4.3.3 displays the antimicrobial activity of MC against *L. monocytogenes*. Aligning with the results of both the *Salmonella* and the *E. coli* O157:H7 antimicrobial activity tests, at each contact time, the population of *L. monocytogenes* significantly decreased as the concentration of MC solution increased (Figure 4.3.3). At 10 min of contact time, bacterial reduction in *L. monocytogenes* increased from 0.79 log in the control group to 3.60 log in the 0.01% MC solution. A complete kill (8.15 log reduction) was obtained in the 0.05% MC solution. Bacteria reduction increased from less than 1 log in the control group to a complete kill in 0.01% MC solution for both the 30 and 60 min contact times.

As seen in the previous experiments, the antimicrobial efficacy of MC also increased with longer contact time (Figure 4.3.3). The control group did not show significant differences in *L. monocytogenes* reduction at the different contact times. At a concentration of 0.01%, the biocidal activity of MC increased steadily, until the solution was able to completely kill all

inoculated bacteria at 30 min of contact time. At 10 min of contact time, the 0.05% MC solution inactivated all inoculated *L. monocytogenes*.

In summary, the results of these 3 experiments indicate that the reduction of *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes*, increased with higher concentration of MC as well as with longer contact time. These results align with previous research of Ren (2015).

Table 4.3.3: Log Reduction of *L. monocytogenes* Treated with MC in BPB

MC Concentration	Contact Time (min)			
	5	**10**	**30**	**60**
0.00%	0.74 ± 0.12 ^{aA}	0.79 ± 0.04 ^{aA}	0.83 ± 0.01 ^{aA}	0.83 ± 0.02 ^{aA}
0.01%	2.82 ± 0.02 ^{bA}	3.60 ± 0.33 ^{bB}	8.15± 0.00 ^{bC}	8.15 ± 0.00 ^{bC}
0.05%	4.68 ± 0.07 ^{cA}	8.15± 0.00 ^{cB}	8.15 ± 0.00 ^{bB}	8.15 ± 0.00 ^{bB}

* Inoculum: 8.15 Log CFU/mL
a,b,c –same character means no difference within a column ($p<0.05$)
A,B,C – same character means no difference within a row ($p<0.05$)

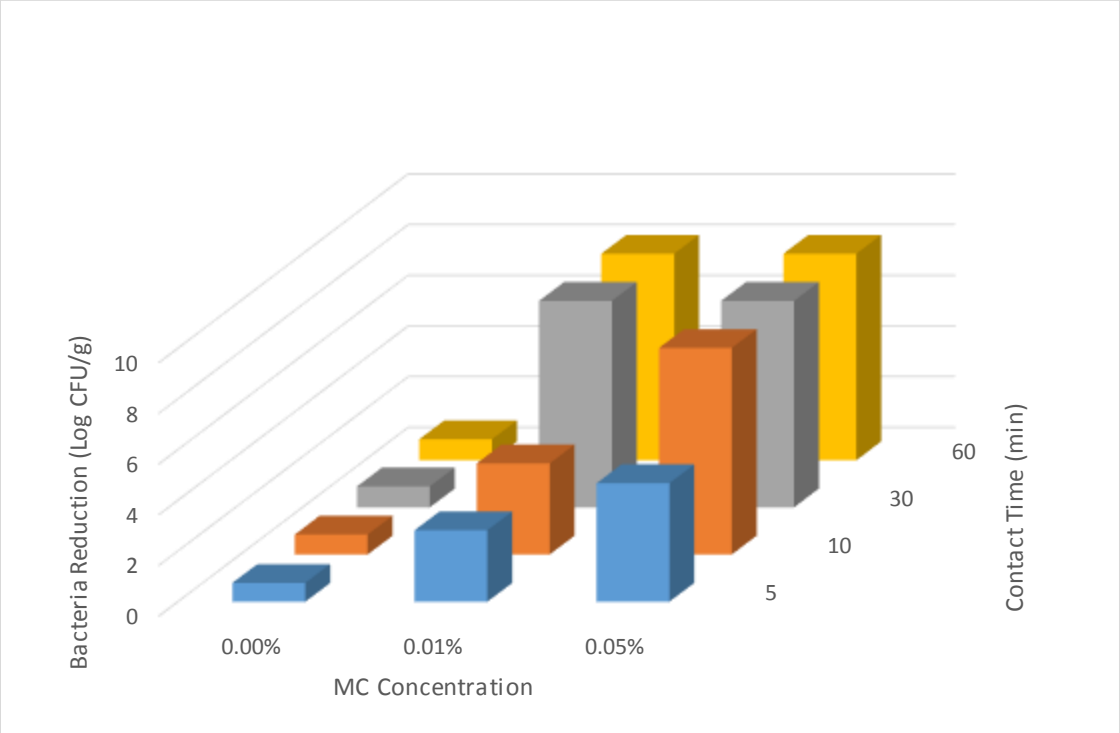

Figure 4.3.3: Antimicrobial activity of MC against *L. monocytogenes* in BPB

4.4: Antimicrobial Efficacy of MC on Microbial Inoculated Alfalfa Sprouts

Table 4.4.1 displays the results of the biocidal activity of MC against inoculated *Salmonella* on alfalfa sprouts. Compared with the control group and the 0.01% MC concentration, the 0.05% MC solution produced the best antimicrobial activity. At each contact time, with an increase in MC concentration, the percentage of *Salmonella* reduction increased (Figure 4.4.1). At 60 min of contact time, the 0.05% MC treatment resulted in a *Salmonella* reduction of only 98.73%. These results are similar to published data which report that the treatment of mature sprouts with surface sanitizing agents to eliminate pathogenic *Salmonella* results in a limited decrease in the population of the target pathogen (Gandhi and Matthews 2003). The lack of treatment efficacy may be due to the location of the pathogen in the inner tissue of the plant.

The reduction of *Salmonella* also increased with longer contact time between the bacteria and the MC (Figure 4.4.1). The control group did show differences in *Salmonella* reduction at different contact times, however, for the MC treated groups, the biocidal activity was more extreme with longer contact times. The control group is essentially a water treatment, indicating that water-washing has limited effectiveness at reducing bacterial populations (Kim and others 2009). In the 0.01% MC treatment, *Salmonella* was significantly reduced from 8.39% to 74.48%, when the contact time was extended from 5 min to 60 min. A similar trend was seen in the 0.05% MC solution, resulting in a 90.21% bacterial reduction at 30 min of contact and a 98.73% *Salmonella* reduction at 60 min of contact. Even with the increased reduction of *Salmonella* with longer contact time between the bacteria and the MC, the overall bacterial reduction was limited.

Table 4.4.1: Reduction (%) of *Salmonella* in MC Treated Alfalfa Sprouts

MC Concentration	Contact Time (min)			
	5	**10**	**30**	**60**
0.00%	11.89 ± 5.93 ^{aA}	16.08 ± 7.91 ^{aAB}	38.46 ± 7.91 ^{aB}	41.96 ± 2.97 ^{aB}
0.01%	8.39 ± 12.36 ^{aA}	9.09 ± 27.20 ^{aAB}	40.91 ± 7.41 ^{aAB}	74.48 ± 0.49 ^{bB}
0.05%	46.50 ± 2.47 ^{bA}	55.24 ± 19.78 ^{aAB}	90.21 ± 0.99 ^{bBC}	98.73 ± 0.57 ^{cC}

* Inoculum: 1.4 x 10^6 CFU/g
a,b,c – same character means no difference within a column ($p<0.05$)
A,B,C – same character means no difference within a row ($p<0.05$)

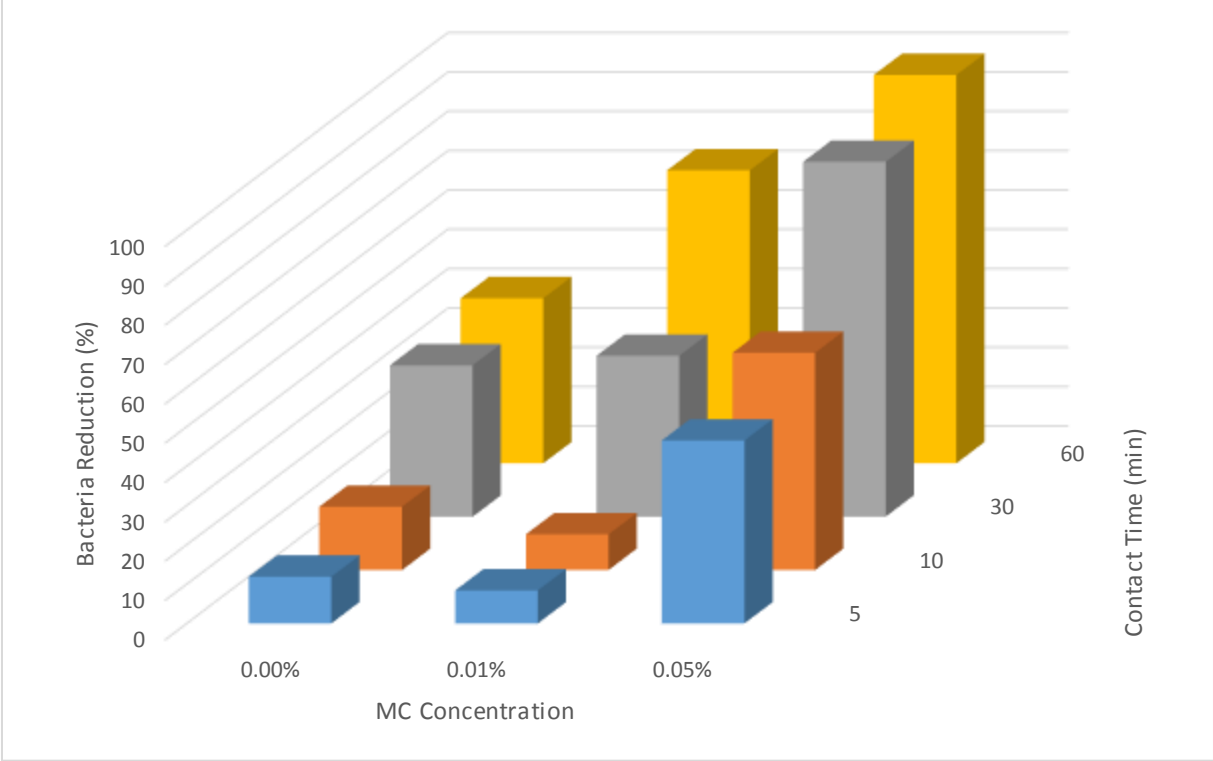

Figure 4.4.1: Antimicrobial activity of MC against inoculated *Salmonella* on alfalfa sprouts

Table 4.4.2 shows the results of the antimicrobial activity of MC against *E. coli* O157:H7 inoculated alfalfa sprouts. The percent reductions were slightly different from the *Salmonella* trial, but the overall trends of bacteria reduction were similar. Compared with the control group and the 0.01% MC treatment, the 0.05% MC treatment showed better antimicrobial activity. At each contact time, the percentage of *E. coli* O157:H7 reduction significantly increased when comparing the control group to the 0.05% MC solution (Figure 4.4.2). As with the results from the *Salmonella* trial, the overall bacterial reduction of *E. coli* O157:H7 was limited. At 60 min of contact time, the 0.01% MC solution resulted in only a 54.63% bacterial reduction, while the 0.05% MC solution eliminated 97.65% of the inoculated bacteria.

The reduction of *E. coli* O157:H7 also increased with longer contact time between the bacteria and the MC (Figure 4.4.2). The control group did not show any significant difference in *E. coli* O157:H7 reduction at the different contact times. These results indicate that soaking alfalfa sprouts with water produces minimal bacterial reductions. In the 0.01% MC solution, *E. coli* O157:H7 reductions at 5, 10, and 30 min of contact time were not significantly different, which were 25.43%, 36.13%, and 36.42%, respectively. In the 0.05% MC treatment, the bacterial reductions were increased with the increase in contact time and it was 97.65% reduction at the 60 min contact time. As previously mentioned, even with the increased reduction of *E. coli* O157:H7 with longer contact time between the bacteria and the MC, the overall bacterial reduction was marginal.

Table 4.4.2: Reduction (%) of *E. coli* O157:H7 in MC Treated Alfalfa Sprouts

MC Concentration	Contact Time (min)			
	5	**10**	**30**	**60**
0.00%	17.34 ± 2.45 ^{aA}	0.58 ± 10.22 ^{aA}	4.34 ± 10.22 ^{aA}	31.79 ± 4.90 ^{aA}
0.01%	25.43 ± 0.82 ^{abA}	36.13 ± 2.86 ^{bAB}	36.42 ± 1.63 ^{bAB}	54.63 ± 8.58 ^{aB}
0.05%	32.37 ± 4.90 ^{bA}	68.50 ± 2.86 ^{cB}	87.86 ± 0.00 ^{cC}	97.65 ± 1.39 ^{bC}

* Inoculum: 1.7 x 10^6 CFU/g
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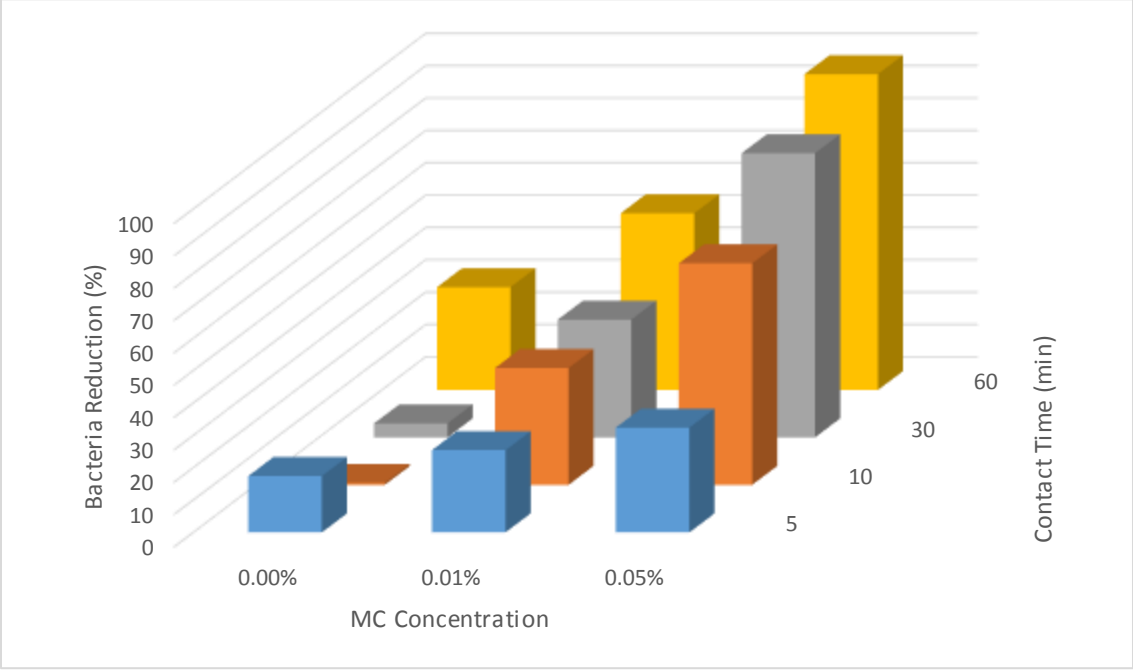

Figure 4.4.2: Antimicrobial activity of MC against inoculated *E. coli* O157:H7 on alfalfa sprouts

Table 4.4.3 shows the results of the antimicrobial activity of MC against *Listeria monocytogenes* inoculated alfalfa sprouts. At each contact time, no significant differences were observed in the percent reduction of *L. monocytogenes* in all treatments. (Figure 4.4.3). However, a significant reduction was seen in the *L. monocytogenes* population when the contact time between the bacteria and the MC was extended (Figure 4.4.3). As in the *E. coli* O157:H7 trial, the control group did not show any significant difference in *L. monocytogenes* reduction at the different contact times. It illustrated that soaking alfalfa sprouts with only water produces minimal bacterial reductions. In the 0.01% MC treatment, bacterial reductions increased with the increase in contact time. However, significant reductions were not seen after 10 min of contact time. The 0.05% MC treatment resulted in a significant increase in biocidal effects as the contact time increased. At 60 min of contact time, a 98.37% reduction of the inoculated *L. monocytogenes* was achieved. However, the overall bacterial reduction of *L. monocytogenes* across time and across MC concentrations was limited.

In summary, the results of these 3 experiments indicate that the percent reductions of *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* inoculated mature alfalfa sprouts, increased with higher concentration of MC as well as with longer contact time between the MC and the bacteria. However, in all 3 trials, the reductions were low due to the physical structure of alfalfa sprouts sheltering the inoculated pathogens from the biocidal effects of the MC (FDA 2015[b]; Gandhi and Matthews 2003). These results suggest that measures including hurdle technology, good agricultural practices (GAP), good manufacturing practices (GMP), and Hazard Analysis and Critical Control Points (HACCP) should be in continual use to reduce the risk of microbial contamination, in mature sprouts, from farm to fork.

Table 4.4.3: Reduction (%) of *L. monocytogenes* in MC Treated Alfalfa Sprouts

MC Concentration	Contact Time (min)			
	5	**10**	**30**	**60**
0.00%	14.78 ± 9.84 ^{aA}	43.48 ± 6.15 ^{aA}	40.43 ± 22.75 ^{aA}	66.52 ± 14.14 ^{aA}
0.01%	20.87 ± 4.92 ^{aA}	58.70 ± 12.91 ^{aB}	68.26 ± 1.84 ^{aB}	86.96 ± 2.46 ^{aB}
0.05%	32.17 ± 19.68 ^{aA}	46.09 ± 3.69 ^{aAB}	85.65 ± 6.76 ^{aBC}	98.37 ± 0.17 ^{aC}

* Inoculum: 1.2 x 10^6 CFU/g
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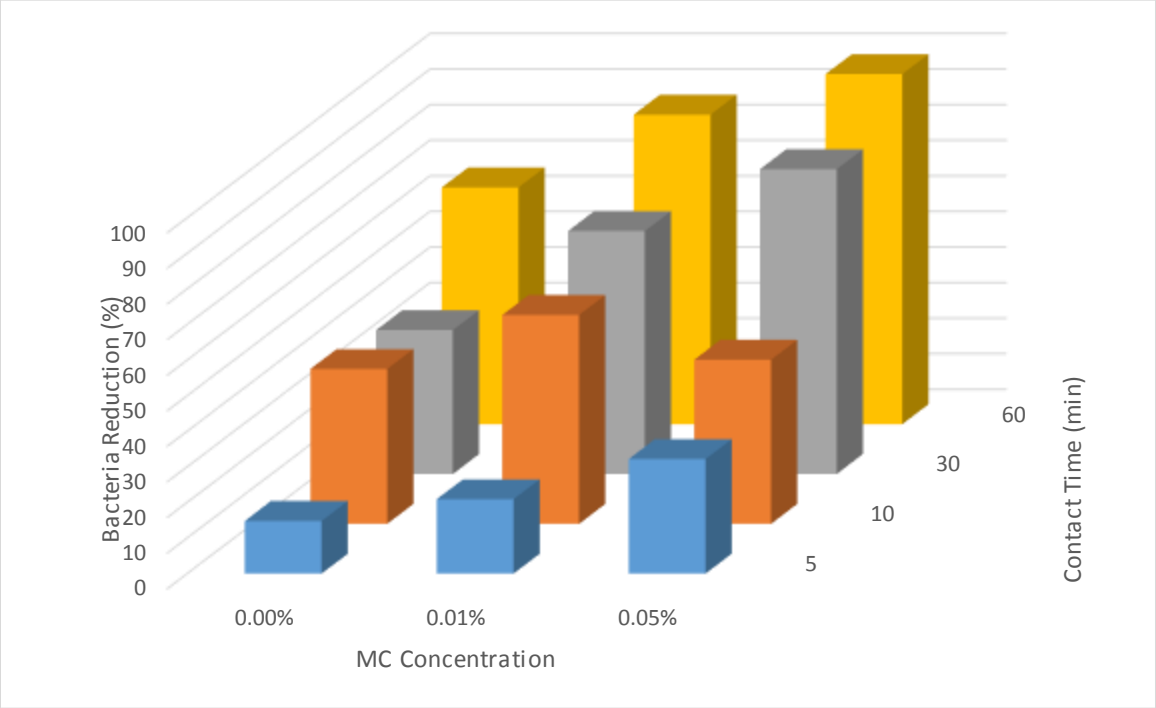

Figure 4.4.3: Antimicrobial activity of MC against inoculated *L. monocytogenes* on alfalfa sprouts

4.5: Longevity of MC's antimicrobial activity

The microbial quality of alfalfa sprouts treated with 0.01% and 0.05% MC then stored at refrigeration temperature was evaluated over the length of 11 d. No significant differences were observed in the microbial quality of the alfalfa sprouts within MC treatment groups from day 0 through day 11. These results indicate that the MC treatments did not improve the microbial quality of the sprouts. Similarly, from days 0 to 11, no significant differences was were found when analyzing the total aerobic bacterial load of the treated sprouts within each day. Based on the results, increasing the concentration of MC does not influence the microbial quality of the alfalfa sprouts during storage.

Table 4.5.1: Aerobic Bacterial Population (Log CFU/g) of Alfalfa Sprouts over 11 days (8 °C)

	MC Concentration		
Day	**0%**	**0.01%**	**0.05%**
0	8.73 ± 0.21 ^{aA}	8.71 ± 0.16 ^{aA}	8.72 ± 0.57 ^{aA}
1	8.50 ± 0.23 ^{aA}	8.68 ± 0.01 ^{aA}	8.42 ± 0.16 ^{aA}
3	8.47 ± 0.16 ^{aA}	8.54 ± 0.16 ^{aA}	8.46 ± 0.43 ^{aA}
5	8.72 ± 0.28 ^{aA}	8.68 ± 0.24 ^{aA}	8.42 ± 0.47 ^{aA}
7	8.61 ± 0.08 ^{aA}	8.61 ± 0.07 ^{aA}	8.46 ± 0.07 ^{aA}
9	8.77 ± 0.08 ^{aA}	8.67 ± 0.11 ^{aA}	8.70 ± 0.06 ^{aA}
11	8.78 ± 0.02 ^{aA}	8.71 ± 0.13 ^{aA}	8.82 ± 0.05 ^{aA}

a,b,c – same character means no difference within a column ($p<0.05$)
A,B,C – same character means no difference within a row ($p<0.05$)

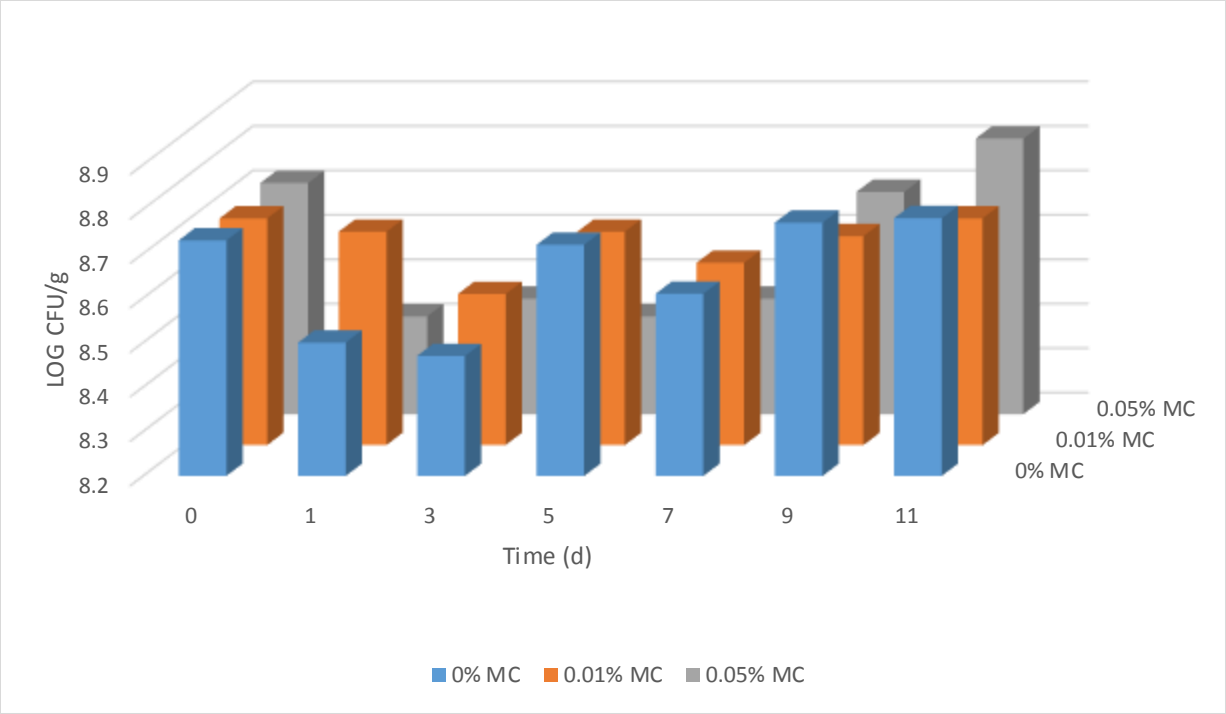

Figure 4.5.1: Aerobic bacterial population (Log CFU/g) of alfalfa sprouts over 11 Days (8 °C)

4.6: MC Effect on Microbial Change in Water for Sprouting

The microbial change in water used for sprouting alfalfa seeds was analyzed over a period of 5 d. Excluding day 4, the microbial load of the water for sprouting among these days were not significantly different. The results from day 4 show a significant difference in the microbial loads with the 0% MC without a daily change and the 0.05% MC with daily change treatments were lower. Further trials need to be completed to investigate the trend, but the frequency of water changes and the addition of MC may have an effect on the overall microbial load of sprouting water, especially during the later days of growth.

When analyzing the microbial load of the water for each MC treatment group, no significant differences were found in the microbial load of the sprouts that did not receive a daily

water change (Table 4.6.1). The sprouts that did receive a daily water change produced significant decreases in the microbial load of the water. These results further illustrate that changing the water daily for sprouting may have benefits hurdle in maintaining the microbial safety of sprouts. Even with the previously mentioned differences in water quality, the final microbial loads of the harvested sprouts were not significantly different among treatments.

Table 4.6.1: Aerobic Bacterial Population (Log CFU/mL) in Alfalfa Sprouting Water Over 5 Days

Day	Water Treatment			
	0% MC with Change	**0% MC w/o Change**	**0.05% MC w/o Change**	**0.05% MC with Change**
0	0 ± 0.00 ^{A}	0 ± 0.00 ^{A}	0 ± 0.00 ^{A}	0 ± 0.00 ^{A}
1	8.38 ± 0.30 ^{aA}	8.60 ± 0.30 ^{aA}	8.81 ± 0.42 ^{aA}	8.66 ± 0.16 ^{aA}
2	7.79 ± 0.22 ^{abA}	8.24 ± 0.38 ^{aA}	8.59 ± 0.12 ^{aA}	7.68 ± 0.05 ^{abA}
3	7.56 ± 0.40 ^{abA}	8.10 ± 0.31 ^{aA}	8.04 ± 0.58 ^{aA}	7.36 ± 0.52 ^{abA}
4	7.12 ± 0.06 ^{bAB}	8.20 ± 0.45 ^{aA}	7.58 ± 0.15 ^{aAB}	6.96 ± 0.28 ^{bB}
5	7.06 ± 0.16 ^{bA}	8.31 ± 0.46 ^{aA}	7.58 ± 0.15 ^{aA}	7.57 ± 0.54 ^{abA}
Day 5 Sprout (Log CFU/g)	8.60 ± 0.21 ^{A}	8.70 ± 0.06 ^{A}	8.63 ± 0.17 ^{A}	8.77 ± 0.02 ^{A}

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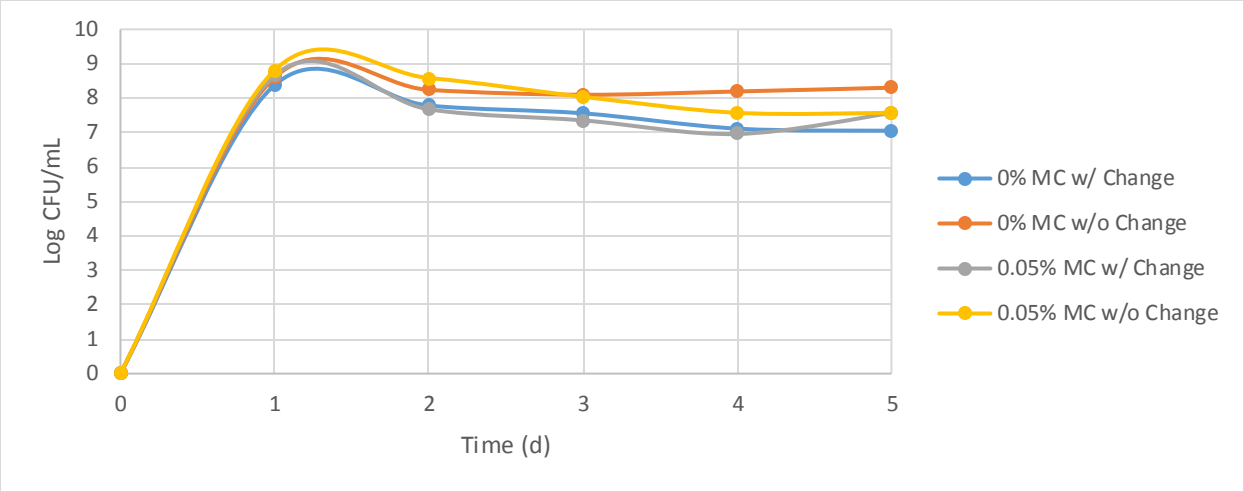

Figure 4.6.1: Aerobic bacterial population (Log CFU/mL) in alfalfa sprouting water over 5 days

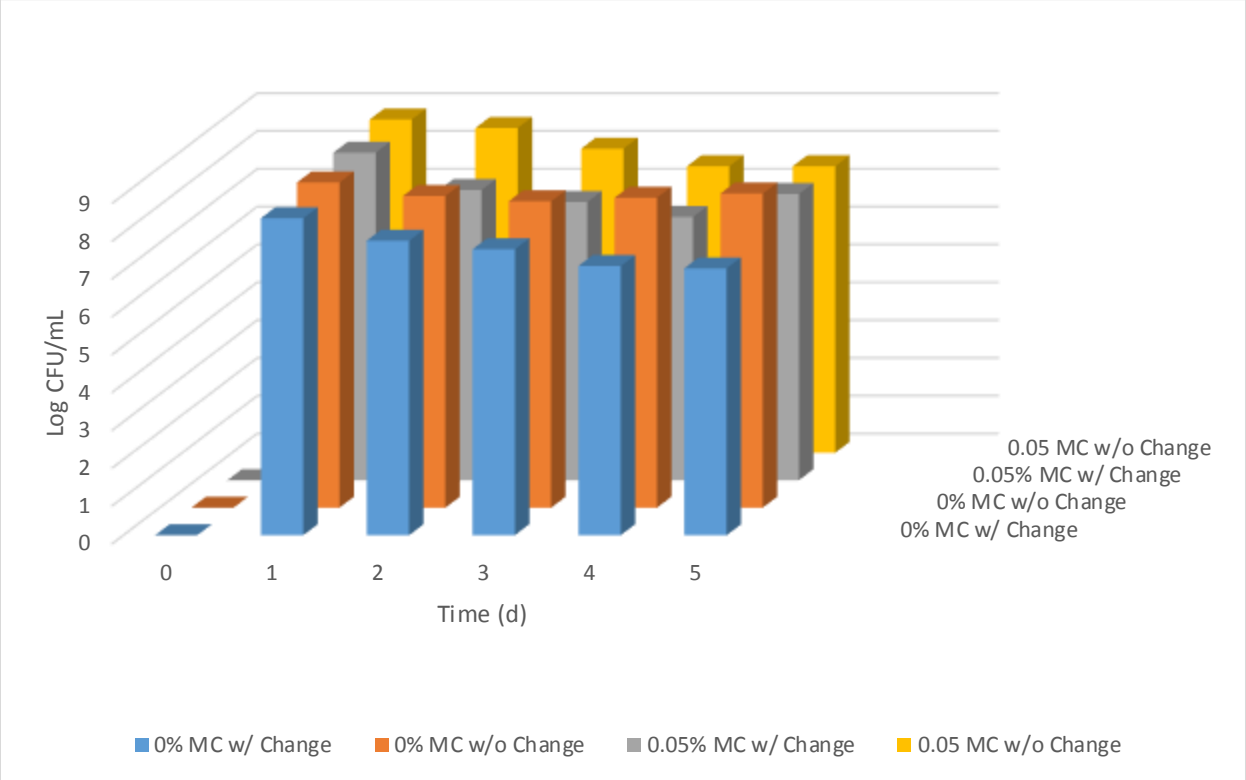

Figure 4.6.2: Aerobic bacterial population (Log CFU/mL) in alfalfa sprouting water over 5 days

Chapter 5: Conclusions

The purpose of this study was to evaluate the antimicrobial activity of MC and its effectiveness at reducing foodborne pathogens on alfalfa sprouts. We also investigated whether MC is a commercially applicable intervention strategy for the control of pathogens in raw sprouts.

In the MC antimicrobial test, we found that at a higher MC concentration or with longer contact time, the biocidal activity of MC against *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* increased. Solutions containing 0.05% MC were able to completely inactivate the inoculated *Salmonella* and *L. monocytogenes* at 10^8 CFU/mL within 10 min of contact time. At the same MC concentration, all inoculated *E. coli* O157:H7 at 10^8 CFU/mL was inactivated within 30 min. Additionally, solutions of 0.01% MC were able to kill all inoculated *L. monocytogenes* and *Salmonella* in 30 min and 60 min of contact times, respectively, and more than 6 logs of the inoculated *E. coli* O157:H7 within 60 min.

When we investigated the antimicrobial activity of MC on inoculated sprouts, results showed that MC was able to significantly reduce bacterial populations, however, overall antimicrobial activities were minimal. Solutions containing 0.05% MC resulted in a less than 2 log reduction in the population of *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes*, within 60 min of contact time. The reductions were minimal due to the physical structure of alfalfa sprouts sheltering pathogens from the biocidal effects of the compound.

This application of MC was not found to compromise the economical integrity of mature alfalfa sprouts. No significant difference was found between the germination rates of alfalfa sprouts treated with MC and those that were not. In addition, the use of MC did not affect the microbial quality of alfalfa sprouts during storage. Some significant differences were found

between the water quality of sprouts treated with MC and those that were not and between those sprouts given daily water changes and those that were not. However, the final microbial loads of the harvested sprouts were not significantly different among treatments.

Our results indicate that MC is a promising antimicrobial agent, nonetheless, further research is warranted to discover how to effectively utilize the compound to inactivate sheltered pathogens in sprouts to enhance their microbial safety.

References

Ariefdjohan M, Nelson P, Singh R, Bhunia A, Balasubramaniam V, Singh N. 2004. Efficacy of high hydrostatic pressure treatment in reducing *Escherichia coli* O157 and *Listeria monocytogenes* in alfalfa seeds. J Food Sci 69:M117-20.

Bari ML, Al-Haq MI, Kawasaki T, Nakauma M, Todoriki S, Kawamoto S, Isshikii K. 2004. Irradiation to kill *Escherichia coli* O157:H7 and *Salmonella* on ready-to-eat radish and mung bean sprouts. J Food Prot 67:2263-8.

Bari ML, Ukuku D, Kawasaki T, Inatsu Y, Isshiki K, Kawamoto S. 2005. Combined efficacy of nisin and pediocin with sodium lactate, citric acid, phytic acid, and potassium sorbate and EDTA in reducing the *Listeria monocytogenes* population of inoculated fresh-cut produce. J Food Prot 68:1381-7.

Belabre B, Dekevich D, DeMent J. 2015. A food production wiki for public health professionals: sprouts. Available from: http://fsi.colostate.edu/sprouts/. Accessed 2016 January 13.

Bennik MJ, Van Overbeek W, Smid EJ, Gorris LM. 1999. Biopreservation in modified atmosphere stored mung bean sprouts: the use of vegetable-associated bacteriocinogenic lactic acid bacteria to control the growth of *Listeria monocytogenes*. Lett Appl Microbiol 28:226-32.

Blatchley ER, Peel MM. 2001. Disinfection by ultraviolet irradiation. In: S.S. Block, editor. Disinfection, sterilization, and preservation. Philadelphia: Lippincott Williams & Wilkins. p. 823 – 52.

Bridges MA, Palczewski CM, Scott JR, Suess J, Nichols EJ, inventors. 2013. Methods and compositions for treating fish diseases. U.S. Patent 8563587.

CDC. 2016[a]. Multistate outbreak of *Salmonella* Muenchen infections linked to alfalfa sprouts produced by sweetwater farms. Available from: http://www.cdc.gov/salmonella/muenchen-02-16/. Accessed 2016 February 26.

CDC. 2016[b]. Multistate outbreak of shiga toxin-producing *Escherichia coli* O157 infections linked to alfalfa sprouts produced by Jack & the Green Sprouts. Available from: http://www.cdc.gov/ecoli/2016/o157-02-16/. Accessed 2016 February 26.

CDC. 2015[a]. *Escherichia coli*. Available from: http://www.cdc.gov/ecoli/. Accessed 2016 February 26.

CDC. 2015[b]. *Salmonella*. Available from: http://www.cdc.gov/salmonella/. Accessed 2016 March 1.

CDC. 2015[c]. *Listeria* (Listeriosis). Available from: http://www.cdc.gov/listeria/. Accessed 2016 March 1.

CDC. 2012. Multistate outbreak of listeriosis linked to whole cantaloupes from Jensen Farms, Colorado. Available from: http://www.cdc.gov/listeria/outbreaks/cantaloupes-jensen-farms/index.html. Accessed 2016 March 1.

Chen Y, Worley SD, Kim J, Wei CI, Chen TY, Santiago JI, Williams JF, Sun G. 2003. Biocidal poly(styrenehydantoin) beads for disinfection of water. Ind Eng Chem Res 42:280-4.

Cherry JP. 1999. Improving the safety of fresh produce with antimicrobials. Food Technol – Chicago 53:54-9.

Cobo Molinos A, Abriouel H, Lopez RL, Valdivia E, Omar NB, Galvez A. 2008. Combined physio-chemical treatments based on enterocin AS-48 for inactivation of gram-negative bacteria in soybean sprouts. Food Chem Toxicol 46:2912-21.

Cobo Molinos A, Abriouel H, Omar NB, Valdivia E, Lopez RL, Maqueda M, Canamero MM, Galvez A. 2005. Effect of immersion solutions containing enterocin AS-48 on *Listeria monocytogenes* in vegetable foods. Appl Environ Microb 71:7781-7.

Demir B, Cerkez I, Worley SD, Broughton RM, Huang TS. 2015. *N* – halamine modified antimicrobial polypropylene nonwoven fabrics for use against airborne bacteria. ACS Appl Mater Interfaces 7:1752-7.

Ding H, Fu TJ, Smith MA. 2013. Microbial contamination in sprouts: how effective is seed disinfection treatment? J Food Sci 78:495-201.

Elder ED, Reid JC. 1993. Potential hydroponic application of an organic *N* – halamine disinfectant. Georgia Water Resources Conference; Athens, GA; 20-21 April 1993. Atlanta, GA: Georgia Institute of Technology Institute of Natural Resources.

Farhangi M, Valadon L. 2006. Effect of acidified processing and storage on carotenoids (provitamin A) and vitamin C in mung bean sprouts. J Food Sci 46:1464-6.

Farkas DF, Hoover DG. 2000. High pressure processing. J Food Sci 65:47-64.

FDA. 2015[a]. Produce safety standards. Available from: http://www.fda.gov/Food/GuidanceRegulation/FSMA/ucm304045.htm. Accessed 2016 January 29.

FDA. 2015[b]. Microbiological safety evaluations and recommendations on sprouted seed. Available from: http://www.fda.gov/Food/FoodborneIllnessContaminants/BuyStoreServeSafeFood/ucm078789.htm. Accessed 2016 January 30.

FDA. 2015[c]. Bad Bug Book – *Salmonella* spp. Available from: http://www.fda.gov/food/foodborneillnesscontaminants/causesofillnessbadbugbook/ucm069966.htm. Accessed 2016 February 5.

FDA. 2014. Bad Bug Book – *Escherichia coli* O157:H7. Available from: http://www.fda.gov/Food/FoodborneIllnessContaminants/CausesOfIllnessBadBugBook/ucm071284.htm. Accessed 2016 February 5.

FDA. 2004. Growing sprouts in retail food establishment. Available from: http://www.fda.gov/Food/GuidanceRegulation/RetailFoodProtection/ucm078758.htm. Accessed 2016 February 6.

Foodborne Illness Outbreak Database. 2016. Available from: http://www.outbreakdatabase.com/search/?vehicle=sprout. Accessed 2016 January 5.

Fransisca L, Zhou B, Park H, Feng H. 2011. The effect of calcinated calcium and chlorine treatments on *Escherichia coli* O157:H7 87-23 population reduction in radish sprouts. J Food Sci 76:M404-12.

Gandhi M, Matthews KR. 2003. Efficacy of chlorine and calcinated calium treatment of alfalfa seeds and sprouts to eliminate *Salmonella*. Int J Food Microbiol 87:301-6.

Hati S, Mandal S, Minz PS, Vij S, Khetra Y, Singh BP, Yadav D. 2012. Electrolyzed oxidized water (EOW): non – thermal approach for decontamination of food borne microorganisms in food industry. Food Nutr Sci 3:760-8.

Hui F, Debiemme-Chouvy C. 2013. Antimicrobial *N* – halamine polymers and coatings: a review of their synthesis, characterization, and applications. Biomacromolecules 14:585-601.

Ikeda T, Yamaguchi H, Tazuke S. 1984. New polymeric biocides: synthesis and antimicrobial activities of polycations with pendant biguanide groups. Antimicrob Agents Chemother 26:139-44.

Jung WY, Choi YM, Rhee, MS. 2009. Potential use of supercritical carbon dioxide to decontaminate *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* typhimurium in alfalfa sprouted seeds. Int J Food Microbiol 136:66-70.

Karch H, Denamur E, Dobrindt U, Finlay BB, Hengge R, Johannes L, Ron EZ, Tonjum T, Sansonetti PJ, Vicente M. 2012. The enemy within us: lessons from the 2011 European *Escherichia coli* O104:H4 outbreak. EMBO Mol Med 4:841-8.

Kenawy ER, Worley SD, Broughton R. 2007. The chemistry and applications of antimicrobial polymers: a state-of-the-art review. Biomacromolecules 8:1359-84.

Khadre M, Yousef A. 2001. Sporicidal action of ozone and hydrogen peroxide: a comparative study. Int J Food Microbiol 71:131-8.

Kim HJ, Feng H, Kushad MM, Fan X. 2006. Effects of ultrasound, irradiation, and acidic electrolyzed water on germination of alfalfa and broccoli seeds and *Escherichia coli* O157:H7. J Food Sci 71:M168-73.

Kim C, Hung YC, Brackett RE, Lin CS. 2003. Efficacy of electrolyzed oxidizing water in inactivating *Salmonella* on alfalfa seeds and sprouts. J Food Prot 66:208-14.

Kim H, Kim H, Bang J, Beuchat LR, Ryu JH. 2010. Synergistic effect of chlorine dioxide and drying treatments for inactivating *Escherichia coli* O157:H7 on radish seeds. J Food Prot 73:1225-30.

Kim JG, Yousef AE, Dave S. 1999. Application of ozone for enhancing the microbiological safety and quality of foods: a review. J Food Prot 62:1071-87.

Kim Y, Kim M, Song KB. 2009. Combined treatment of fumaric acid with aqueous chlorine dioxide or UV-C irradiation to inactivate *Escherichia coli* O157:H7, *Salmonella enterica* serovar Typhimurium, and *Listeria monocytogenes* inoculated on alfalfa clover sprouts. Food Sci Technol 42:1654-8.

Kocharunchitt C, Ross T, McNeil D. 2009. Use of bacteriophages as biocontrol agents to control *Salmonella* associated with seed sprouts. Int J Food Microbiol 128:453-9.

Li C, Xue L, Cai Q, Bao S, Zhao T, Xiao L, Gao G, Harnoode C, Doug A. 2014. Design, synthesis and biocidal effect of novel amine *N* – halamine microspheres based on 2,2,6,6-tetramethyl-4-piperidinol as promising antimicrobial agents. RSC Adv 4:47853-64.

Melero B, Vinuesa R, Diez AM, Jaime I, Rovira J. 2012. Application of protective cultures against *Listeria monocytogenes* and *Campylobacter jejuni* in chicken products packaged under modified atmosphere. Poult Sci 92: 1108-16.

Montville R, Schaffner DW. 2003. Analysis of published sprout seed sanitization studies shows treatments are highly variable. J Food Prot 67:758-65.

Olaimat AN, Holley RA. 2012. Factors influencing the microbial safety of fresh produce: a review. Food Microbiol 32:1-19.

Pao S, Kalantari A, Khalid M. 2008. Eliminating *Salmonella enterica* in alfalfa and mung bean sprouts by organic acid and hot water immersions. J Food Process Preserv 32:335-42.

Pao S, Rolph S, Westbrook E, Shen H. 2004. Use of bacteriophages to control *Salmonella* in experimentally contaminated sprout seeds. J Food Sci 69:M127-30.

Parent RA. 2000. Acute toxicity data submissions. Int J Toxicol 19:331-73.

Pui CF, Wong WC, Chai LC, Tunung R, Jeyaletchumi P, Noor Hidayah MS, Ubong A, Farinazleen MG, Cheah YK, Son R. 2011. *Salmonella*: A foodborne pathogen. Int Food Res J 18:465-73.

Quintavalla S, Vicini L. 2002. Antimicrobial food packaging in meat industry. Meat Sci 62:373-80.

Roberts RG, Reymond ST. 1994. Chlorine dioxide for reduction of postharvest pathogen inoculum during handling of tree fruits. Appl Environ Microbiol 60:2864-8.

Scharff RL. 2011. Economic burden from health losses due to foodborne illness in the United States. J Food Prot 75: 123-31.

Settanni L, Corsetti A. 2008. Application of bacteriocins in vegetable food biopreservation. Int J Food Microbiol 121:123-38.

Seo YH, Jang JH, Moon, KD. 2010. Occurrence and characterization of enterotoxigenic *Staphylococcus aureus* isolated from minimally processed vegetables and sprouts in Korea. Food Sci Biotechnol 19:313-9.

Siedenbiedel F, Tiller JC. 2012. Antimicrobial polymers in solution and on surfaces: overview and functional principles. Polymers 4:46-71.

Sikin A, Zoellner C, Rizvi S. 2013. Current intervention strategies for the microbial safety of sprouts. J Food Prot 76:2099-123.

Singla R, Ganguli A, Ghosh M. 2011. An effective combined treatment using malic acid and ozone inhibits *Shigella* spp. on sprouts. Food Control 22:1032-9.

Soylemez G, Brashears MM, Smith DA, Cuppett SL. 2001. Microbial quality of alfalfa seeds and sprouts after a chlorine treatment and packaging modifications. J Food Sci 66:153-7.

Sun G, Wheatley WB, Worley SD. 1994. A new cyclic *N* – halamine biocidal polymer. Ind Eng Chem Res 33:168-70.

Trinetta V, Vaidya N, Linton R, Morgan M. 2011. Evaluation of chlorine dioxide gas residues on selected food produce. J Food Sci 76:T11-5.

USDA. 2015. *Escherichia coli* O157:H7 and other shiga toxin-producing *E. coli* (STEC). Available from: http://www.fsis.usda.gov/wps/portal/fsis/topics/food-safety-education/get-answers/food-safety-fact-sheets/foodborne-illness-and-disease/escherichia-coli-o157h7/ct_index. Accessed 2016 March 1.

USDA. 2016[a]. *Listeria monocytogenes*. Available from: http://www.fsis.usda.gov/wps/portal/fsis/topics/food-safety-education/get-answers/food-safety-fact-sheets/foodborne-illness-and-disease/listeria-monocytogenes/listeria-monocytogenes. Accessed 2016 March 1.

USDA. 2016[b]. NACMCF Reports and Recommendations. Available from: http://www.fsis.usda.gov/wps/portal/fsis/topics/regulations/advisory-committees/nacmcf-reports/nacmcf-reports. Accessed 2016 February 28.

Wade WN, Scouten AJ, McWatters KH, Wick RL, Demirci A, Fett WF, Beuchat LR. 2003. Efficacy of ozone in killing *Listeria monocytogenes* on alfalfa seeds and sprouts and effects on sensory quality of sprouts. J Food Prot 66:44-51.

Weiss A, Hammes WP. 2005. Efficacy of heat treatment in the reduction of salmonellae and *Escherichia coli* O157:H- on alfalfa, mung bean and radish seeds used for sprout production. Eur Food Res Technol 221:187-91.

Wu L, Ashraf M, Racci M, Wang R, Paterson PG, Ferrie A, Juurlink, BJ. 2004. Dietary approach to attenuate oxidative stress, hypertension, and inflammation in the cardiovascular system. Proc. Natl. Acad. Sci. USA 101:7094-9.

Yaun BR, Sumner SS, Eifert JD, Marcy JE. 2004. Inhibition of pathogens on fresh produce by ultraviolet energy. Int J Food Microbiol 90:1-8.

Ye J, Kostrzynska M, Dunfield K, Warriner K. 2010. Control of *Salmonella* on sprouting mung bean and alfalfa seeds by using a biocontrol preparation based on antagonistic bacteria and lytic bacteriophages. J Food Prot 73:9-17.

Yun J, Li X, Fan X, Li W, Jiang Y. 2013. Growth and quality of soybean sprouts (*Glycine max* L. Merrill) as affected by gamma irradiation. Radiat Phys Chem 82:106-11.